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NATIONAL SURVEILLANCE STRATEGY FOR ANTIMICROBIAL RESISTANCE IN AQUACULTURE

National Surveillance Strategy for Antimicrobial Resistance in Aquaculture

The Fleming Fund Country Grant Pakistan
June, 2024

Foreword

Food security is a key factor in defeating malnutrition and improving a country's socioeconomic status. To overcome nutrition deficiencies, fisheries contribute a significant amount of animal protein to the diets of people worldwide. This source of protein is highly nutritious and the cheapest compared to livestock and poultry. Aquaculture also plays a vital role in national economic development and global food supply. However, this sector may contribute to the spread of antimicrobial resistance, one of the important global one health issues, due to excessive therapeutic and prophylactic antimicrobial use.

To curtail antimicrobial resistance, a country should have a strong monitoring and surveillance system for AMR in the veterinary and human sectors. The Ministry of National Food Security and Research has already developed the “National Surveillance Strategy for AMR in Healthy Food Animals” and “National Surveillance Strategy for AMR in Sick Food Animals” followed by the implementation of a national AMR surveillance pilot in healthy food animals. In order to undermine the driving forces for the inter-sectoral AMR spread, it's monitoring in aquaculture and fisheries is equally important. For the establishment of baseline data on the prevalence of resistant microorganisms, strengthening of AMR surveillance components and genomic analysis for isolated bacteria in aquaculture, the “National Surveillance Strategy for AMR in Aquaculture” has been developed. For this document, all provincial fisheries departments, academic institutes’ fisheries departments, Aquaculture and Fisheries Program NARC, Fisheries Development Board, aquaculture farmers, donor agencies and other relevant stakeholders were consulted.

I congratulate the team for achieving another milestone in the veterinary sector through the support of Fleming Fund Country Grant Pakistan. I am looking forward to the implementation of this strategy in parallel with the strengthening of AMR laboratories for aquaculture in the country.

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Message from Team Lead, Fleming Fund Country Grant Pakistan (DAI)

Antimicrobial use (AMU) in the rapidly expanding aquaculture industry may contribute to the rise of antimicrobial resistance, carrying potential consequences for animal, human, and ecosystem health. Hence, timely detection of antimicrobial-resistant pathogens and continuous monitoring programmes are inevitable. This document will help the authorities to curb the use of antibiotics and implement appropriate management measures to overcome the threat.

Antimicrobial resistance has become a global public health concern. National governments and international organizations recognize the concern and are making efforts to curb and curtail the menace. Since the problem is multifaceted and multidimensional, solutions also need to be multi-pronged. As against the earlier concepts of a narrow focus on the human sector alone, it is now widely recognized that animals and the environment are equally, if not more, important and need to be equally focused in any and all interventions targeting antimicrobial resistance (AMR); the concept named 'One Health Approach.

Fleming Fund, set up by the UK in response to the UK AMR review and WHO Global Action Plan on AMR, has also been built around the same concept. The fund has been supporting the Government of Pakistan since 2019 in its efforts against AMR on all fronts; Human, Veterinary and Environment. The document in hand "National Surveillance Strategy for Antimicrobial Resistance in Aquaculture" has been developed as part of this collaboration. This strategy will help the authorities to control and manage the use of antibiotics in aquaculture. This will have a cross-cutting impact as aquaculture has the potential to contribute towards both human and veterinary sectors.
Fleming Fund Country Grant Pakistan stands with the People and Government of Pakistan in their fight against AMR.

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List of Acronyms

AFP	Aquaculture and Fisheries Program
AHC	Animal Husbandry commissioner
AIM	*Aeromonas* Isolation Medium
AMR	Antimicrobial Resistance
AMU	Antimicrobial Use
ARB	Antimicrobial Resistant Bacteria
AST	Antibiotic Susceptibility Testing
BGA	Brilliant Green Agar
CBP	Clinical susceptibility breakpoints
CLSI	Clinical and Laboratory Standards Institute
ECOFF	Epidemiologic cut-off value
EMB	Eosin Methylene Blue
FAO	Food and Agriculture Organization of the United Nations
FSS	Field Surveillance Site
GLASS	Global Antimicrobial Surveillance System
MIC	Minimum Inhibitory Concentration
MoNFS&R	Ministry of National Food Security and Research
MDR	Multi Drug Resistance
MR-VP	Methyl Red-Voges Proskauer
MT	Metric Tonnes
NAP	National Action Plan
NARC	National Agriculture Research Centre
NFP	National Focal Person
NRL	National Reference Laboratory
NVL	National Veterinary Laboratory
PFP	Provincial Focal Person
PPE	Personal Protective Equipment
PPL	Provincial Peripheral Laboratory
SEA	South East Asia

WHO	World Health Organization
WOAH	World Organization for Animal Health

1. Introduction

Antimicrobial resistance (AMR) is the greatest emerging threat to global public health systems and food security, whereby the microbes that cause diseases are becoming resistant to the antimicrobials used to treat them. Food production environments are considered to pose a particularly high risk to the emergence and dissemination of AMR, especially in low- and middle-income countries (LMICs)[1]. It has been estimated that AMR is responsible for the death of 700,000 humans annually and this number is expected to increase to 10 million lives each year by 2050 in the absence of an effective intervention to contain its emergence and spread[1,2]. In 2024 it was estimated that every year 7·7 million deaths are associated with bacterial infections. Out of the deaths caused by bacterial infections 4·95 million are associated with AMR, of which 1.27 million are caused by bacterial pathogens resistant to the antibiotics available to treat them[3]. The 2024 WHO Bacterial Priority Pathogens List includes 24 antibiotic-resistant bacterial pathogens spanning 15 families. These pathogens are categorized into critical, high, and medium priority groups to guide research, development, and public health interventions in the fight against antimicrobial resistance[4].

Aquaculture is a rapidly growing industry that currently accounts for almost half of the fish used for human consumption worldwide. Intensive and semi-intensive practices are used to maximize fish production volume, but frequent disease outbreaks could occur often requiring antimicrobial use (AMU)[5]. These antimicrobials are usually administered to entire populations containing sick, healthy, and carrier fish, by a process known as metaphylaxis. They are usually administered orally to groups of fish that share tanks or cages, in formulated feed, and occasionally by bath, and by immersion in closed containers. In the absence of collectors to remove uneaten medicated feed from water, it is estimated that up to 80% of the administered antimicrobial active ingredients remain in the water and sediments close to the application sites[2,7]. Therefore, it is expected that the aquaculture environment could retain higher antimicrobial concentrations than those in terrestrial animal farming conditions. The exact AMU levels are not easy to determine because countries have different distribution and registration systems[5] as well as modes of administration and farm production decontamination practices (waste/water disposal from animal environment). For aquatic diseases in Southeast Asia (SEA) resistance to 17 antimicrobial classes has been reported[6]. According to the study, resistance to the following classes were frequently observed: aminoglycosides, beta-lactams, (fluoro)quinolones, tetracyclines and sulfonamides[6]. Additionally, beta-lactam antimicrobials, tetracyclines as well as sulfonamides were observed at levels above 40%[6] in isolates tested. In terms of antimicrobial resistant bacteria (ARB), the indicator Gram-negative organism *Escherichia coli*, and foodborne pathogens *Aeromonas* spp. and *Vibrio* spp. were the most widely and frequently reported ARB in SEA aquaculture sector[6].

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[1]Kelly Thornber, Abul Bashar, Md. Salahuddin Ahmed, Ashley Bell, Jahcub Trew, Mahmudul Hasan, Neaz A. Hasan, Md. Mehedi Alam, Dominique L. Chaput, Mohammad Mahfujul Haque, and Charles R. Tyler, 2022. Antimicrobial Resistance in Aquaculture Environments: Unravelling the Complexity and Connectivity of the Underlying Societal Drivers. Environmental Science & Technology, 56 (21), 14891-14903

[2]O'Neill, J. 2016. Tackling drug-resistant infections globally: final report and recommendations. London: The Review on Antimicrobial Resistance; Available from: https://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf.

[3] GBD 2019 Antimicrobial Resistance Collaborators. Global mortality associated with 33 bacterial pathogens in 2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet. 2022 Dec 17;400(10369):2221-2248. doi: 10.1016/S0140-6736(22)02185-7. Epub 2022 Nov 21. PMID: 36423648; PMCID: PMC9763654.

[4] *The 2024 WHO Bacterial Priority Pathogens List includes 24 antibiotic-resistant bacterial pathogens spanning 15 families. These pathogens are categorized into critical, high, and medium priority groups to guide research, development, and public health interventions in the fight against antimicrobial resistance.*

[5] *Santos L, and F Ramos, 2018. Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. International Journal of Antimicrobial Agents 52 (2018) 135–143.*

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The need for intervention to control AMR in aquaculture is evidenced by the abundant scientific literature that reveals high levels of AMR in aquaculture environments and the associated risks to human, animal, and environmental health, especially in LMICs[1,3,5,6]. Moreover, as the pressure on food security and climate change intensifies, AMR in aquaculture would continue to increase considering that indiscriminate use of antimicrobials is common in many countries, including various top aquatic animal producing countries[9]. The literature reported diverse use of antimicrobial active ingredients, where an average 15 antimicrobial per country are used in the top 15 aquaculture producing countries, of which more than half fall in the category of LMIC[10]. It is also predicted that AMR in aquaculture would primarily impact countries with higher temperatures[9].

An essential measure to minimise the risk of AMR in aquaculture is to monitor AMU at a national, regional, and global level and advocate AMU reduction through better stewardship among involving farmers, veterinarians/aquatic animal health professionals (AAHP) and Veterinary Services (VS)/Aquatic Animal Health Services (AAHS). However, in aquaculture, monitoring AMU is a complicated task due to the diversity of species and culture systems, the unconsolidated nature of production in many regions, and the commonly unregulated use of antimicrobials[11], for example, over-the-counter use and off-label use of antimicrobials. Over 90% of the world aquaculture production is carried out in in LMIC where regulation and enforcement, as well as practices and resources in aquaculture are limited[11]. Furthermore, often antimicrobial treatments are administered without professional consultation or uninformed by any susceptibility testing[16]. Due to the high costs of developing new antibiotic molecules, antibiotic agents used in human and veterinary sector are also used in aquaculture sector. Six common classes of antibiotics (aminoglycosides, macrolides, penicillins, quinolones, sulphonamides, and tetracyclines) that are regularly used in aquaculture (and in livestock) are listed by the World Health Organization (WHO) as critically or highly important antimicrobials[12, 13].

External sources of AMR such as livestock and human wastewater that affect aquaculture environments are also critical and demand rigorous investigation. In LMICs, combining livestock and aquaculture in integrated farming systems presents an option for increased productivity, yet the potential exchange of ARBs and their genes from livestock wastes increases the risk of AMR in those systems[8]. Such a combination of unregulated drug use, intensive fish production and unchecked circulation of ARBs from aquatic animals to humans has created a perfect AMR storm that could lead to serious and long-term consequences. Country and regional programs for the surveillance and monitoring of AMR in bacteria isolated from aquatic animals are necessary, as described in the World Organization of Animal Health (WOAH)'s Aquatic Code[3,14,15].

[8] Cabello F, Godfrey H, Buschmann A, Dölz H. Aquaculture is yet another environmental gateway to the development and globalisation of antimicrobial resistance. Lancet Infect Dis 2016;16: e127–33.
[9]Reverter, M, S Sarter, D Caruso, J-C Avarre, M Combe, E Pepey, L Pouyaud, S Vega-Heredía, H de Verdal, RE Gozlan 2020. Aquaculture at the crossroads of global warming and antimicrobial resistance. Nat Commun 11:1870.
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1.1 Rationale for the Strategy

AMR data from aquaculture production in Pakistan is a knowledge gap in understanding the transmission of zoonotic and foodborne pathogen across the One Health domains (environment, animals, humans). Data from aquatic animals contributes to the "state of science" on AMR in Pakistan and integrated AMU/AMR surveillance initiatives and could be used for further studies including risk analysis of foodborne and zoonotic bacteria from aquatic animals, source attribution and microbial source tracking.

1.2 Purpose of the Strategy

This strategy outlines the framework for AMR surveillance in aquaculture in Pakistan that would serve as a guide in capacity building coordination and technical preparation for development of methods for AMR surveillance programmes in aquaculture production for the purposes of informing public health policy makers. This strategy will be a guiding document for the revision of AMR National Action Plan of Pakistan (2024-2028).

Moreover, the proposed approach for the progressive expansion of the activities from pilot/limited scale to a full systematic national sustainable AMR surveillance programme implementation is the ultimate goal of the current strategy.

1.3 Scope

This document outlines the proposed surveillance framework, methodologies and coordination mechanisms for the surveillance of AMR in aquatic animals in Pakistan.

1.4 Intended Users of this Strategy

This strategy has been developed by MoNFS&R in consultation with relevant public and private stakeholders of aquaculture sector. It is intended for development and integration of aquatic AMR surveillance operationalization. The document is also a guideline for all relevant stakeholders including academia, research institutions and other donors.

2. Current State of AMR in Aquaculture in Pakistan

Pakistan is an agricultural country and is endowed with huge natural water resources, comprised of freshwater, marine and brackish water. According to the Economic Survey of Pakistan, 2023, approximately 193 freshwater fish species, and 800 marine fish species are present in Pakistan. Of these, only 31 of the freshwater fish species were considered commercially important and widely cultured. While 120 marine species (wild-caught) are commercially important, none of the marine species have been known to thrive in an aquaculture environment in Pakistan. Fishing sector having share of 1.39% in agriculture value addition and 0.32% in GDP, grew at 1.44% in 2023 compared to 0.35% in 2022. The doubled growth over a period of a single year without any regulatory framework on antimicrobials use makes Pakistan highly susceptible to the evils of AMR. Yet no concentrated efforts have been conducted on a national level to collect baseline information to evaluate the burden of AMR in the country. Therefore, the current strategy will assess AMR burden in the aquaculture sector of Pakistan to contribute data for source attribution and risk assessment.

3. Framework for AMR Surveillance in aquaculture in Pakistan

This section describes the proposed approach to AMR surveillance in aquaculture and surveillance elements including the scale of statistical representativeness, specimens, microorganisms, and Anti-microbial susceptibility testing (AST) panel to be used. The document is being based on the country's

collective knowledge obtained from previous years during Phase I of Fleming fund Country Grant through surveillance piloting in healthy food animals (livestock and poultry) and lessons learned therein. This framework is the first of its kind being developed for the aquaculture sector to serve as a guide or further discussions with stakeholders and relevant parties involved in the implementation of the surveillance programme.

3.1 Objectives

Through consultation with federal and provincial stakeholders, the following objectives have been identified for the surveillance of AMR in aquaculture:

1. Establish baseline data on the prevalence of bacterial pathogens and their resistance to antimicrobials in select aquatic species raised in various aquaculture production systems.
2. Strengthen AMR surveillance system capacities for aquaculture sector, including epidemiological design (sampling frame, sample size calculation, sampling methodology), sample transportation, laboratory processing and quality data production.
3. Contribute aquaculture AMR data into National AMR surveillance system, and potentially, contribute to global AMR data.
4. Provision of policy guidelines to the concerned authorities based on AMR data in aquaculture of Pakistan.

3.2 Target Bacterial Species

With the world's growing population and potential global trade of aquaculture, the risk of environmental contamination and development of aquatic-derived zoonoses in humans are increasing. Fish derived zoonotic diseases have caused considerable problems in the aquaculture industry and fishery worldwide such as those caused by *Mycobacterium, Escherichia coli, Aeromonas, Salmonella, Streptococcus iniae*[15,16]. The majority of the fish-derived zoonotic diseases are transmitted to humans mainly via the consumption of improperly cooked or raw fish or fish products. Therefore, the incidence of zoonotic diseases can be reduced by properly processing fish and fish products, e.g. by thermal (heat/freezing) treatment. The prevalence of zoonotic agents in fishes varies seasonally and should be regularly monitored[14,16,17]. The Common fish pathogens that infect fish handlers include *Aeromonas hydrophilia*, *Mycobacterium marinum*, *Streptococcus iniae*, *Vibrio vulnificus* and *Photobacterium damselae*.[16] Foodborne illnesses associated with the consumption of fish involve mainly *Listeria monocytogenes*, *Aeromonas* spp. and *Clostridium* spp.[16] Studies have suggested that commercial fish and seafood may act as a reservoir for multidrug-resistant (MDR) bacteria [17]**.** *Salmonella* spp. is among a pathogen of zoonotic importance at point of sale (fish market sampling) for the purposes of assessing food safety of fish products sold for human consumption. CDC estimates that *Salmonella* causes about 1.35 million infections in humans that lead to 26,500 hospitalizations, and 420 deaths in the United States every year[19] . Consumption of *Salmonella*-contaminated fish and their products could cause symptoms such as gastroenteritis, abdominal cramps, fever, and bacteremia. For example, *Salmonella*-infected smoked fish can also serve as a vehicle for foodborne bacteria via contaminated skin, gills, and intestines. *Salmonella*'s persistence in fish intestines and its shedding in feces could end up in the environment and subsequently spread elsewhere. Evidence of concurrent isolation of bacteria belonging to the Enterobacterales such as *E. coli*, *Klebsiella*, and *Salmonella* in fish and people underscores the zoonotic potential of these organisms (including antimicrobial resistant strains)[18]. The most common route of human infection with these bacteria is

[17]1Ziarati M, Zorriehzahra MJ, Hassantabar F, Mehrabi Z, Dhawan M, Sharun K, Emran TB, Dhama K, Chaicumpa W, Shamsi S. Zoonotic diseases of fish and their prevention and control. Vet Q. 2022 Dec;42(1):95-118. doi: 10.1080/01652176.2022.2080298. PMID: 35635057; PMCID: PMC9397527.

[18]Oliviera RV, Oliviera MC, Pelli A.. 2017. Disease infection by Enterobacteriaceae family in fishes: a review. J Microbiol Exp. 4(5):00128

[19] US FDA. *Salmonella*. https://www.fda.gov/animal-veterinary/animal-health-literacy/get-facts-about-salmonella

through open wounds, direct contact via fish/fish products, or scratches serving as entry point of bacteria leading to systemic sequelae.

Based on the above-mentioned studies and technical discussions in the stakeholders meeting (**Annexure I**), the following bacterial species are identified for the national AMR surveillance in aquaculture sector:

- As indicator organism*:*
  - *Escherichia coli*
- As foodborne pathogens*:*
  - *Aeromonas* spp*.*
  - *Salmonella* spp*.*
  - *Vibrio* spp*.*
  - *Flavobacterium* spp*.*
  - *Yersinia* spp*.*
  - *Listeria monocytogenes*

Though the list of bacteria is curated for the purpose of National AMR surveillance, a pilot AMR surveillance will be conducted in the first year proceeding the National efforts in aquaculture. This pilot phase will be conducted to streamline and refine the study design, transportation mechanisms, laboratory procedures along with feasibility testing of the larger and broader surveillance design for the national surveillance efforts.

3.2.1 Priority Pathogens for Pilot AMR Surveillance

Prior to initiation of the national level AMR surveillance, it is imperative that a pilot surveillance is conducted in the first phase of the surveillance in aquaculture. The pilot will not only streamline the AMR surveillance systems in the aquaculture sector, but it will also facilitate the sample size calculations for the National AMR surveillance that would be based on AMR data specific to the unique epidemiological factors driving AMR in Pakistan's aquaculture sector. Therefore, the stakeholders have prioritized the following two bacterial organisms for the pilot study where pond fish sampling will be done with focus on isolation of following bacteria:

- *Aeromonas* spp. (as foodborne pathogen)
- *Escherichia coli* (as indicator organism)

After the pilot phase, the initial data will be presented to relevant stakeholders, One Health AMR committees and policy makers. The surveillance of other organisms will be conducted depending on the initial results and national capacities to implement a full systematic sample collection across the country.

3.3 Sampling Frame

A sampling frame refers to a list of sampling units from which samples can be collected. The sampling frame will be unique for each production system. In the case of an intensive/semi-intensive production system, a farm will be considered as the sampling unit while for the sampling at point-of-sale (grocery stores, wet markets, etc.), a shop in the fish market will be considered as a sampling unit.

The list of sampling frames is generated with the help of federal and provincial aquaculture development departments. Area-wise sampling plan and testing of target bacteria by production systems are given in Table 1 and Table 2 respectively.

For the pilot phase, only intensive/semi-intensive production systems will be targeted where a farm will be considered as a single sampling unit, irrespective of the number of ponds present in the farm.

The method for within-farm pond selection will be simple random sampling that will be conducted by generating random numbers and selecting three to five representative ponds for fish sampling, where one fish/water sample per pond will be selected and then pooled to be identified as one composite sample per farm.

3.4 Sample Size

Based on FAO guidelines on aquaculture one isolate of a specific bacterial type per farm represented in the dataset will be sufficient to estimate the potential AMR on that farm [20]. For each farm three-five fish will be collected and pooled to represent one sample (composite sampling). The number of isolates needed to estimate the prevalence of resistant bacteria are estimated using the following formula:

$$N = [Z2\times (P) \times (1-P)]/e2$$

Where:
N = Total bacterial isolates to be tested per year,
P = Prevalence of the resistance gene or phenotype,
Z = The standard normal deviation, typically set at 95 percent confidence level (z=1.96) and
e = Error (usually five percent or 0.05).

Alternatively, the above calculation can be done using webtools such as OpenEpi as well as Epi Info, that are developed by Rollins School of Public Health at Emory University and CDC respectively. Such tools are user friendly and facilitate sample size calculations after specifying study criteria. An example for calculating sample size is detailed in **Annexure II**.
Assuming 50% of the isolates tested per year will be positive for resistant phenotypes (for example to tetracycline), with 95% level of confidence and 5% desired precision a total of 384 bacterial isolates needs to be recovered for AST for each selected pathogen, both from fish and water separately for National AMR surveillance. As stated earlier, these 384 fish and 384 water samples will be composite in nature i.e. pooling of 3-5 samples will be done from 3-5 ponds per farm. These samples will be collected from the 23 districts that have been identified through stakeholder consultations in the aquaculture sector (Table 1).
The number of farms/point-of-sale shops to be included in the national surveillance study to achieve the isolate size of 384 for sample collection for both types of biological samples (fish and water) is given in **Annexure III**. The pathogens whose prevalence has not been published in literature were assumed to be 50%. These sample sizes will be adjusted based on availability of resources, total farms in the country, and the initial data from pilot phase.

For the pilot phase, we will start with a limited number of sites (eight (n=8)). A total of three to five fish per epidemiological unit/site per month will be collected and pooled to make one sample per site per month. Therefore, each month 8 pooled samples will be available to detect both priority bacteria (*E. coli* and *Aeromonas*) for the duration of the pilot.

3.5 Priority AMR Sampling Sites, Fish Type and Production System

For surveillance targeting bacteria, the aim is to estimate the unbiased national prevalence of AMR at the farm/pond level for different bacteria-antimicrobial combinations (i.e., the proportion of farms that has AMR for the given combination). The most appropriate unit of interest is the farm/pond, as

[20] FAO, NParks and SFA, 2023. Monitoring and surveillance of antimicrobial resistance in bacterial pathogens from aquaculture – Regional Guidelines for the Monitoring and Surveillance of Antimicrobial Resistance, Use and Residues in Food and Agriculture. Volume 3. Bangkok. https://doi.org/10.4060/cc3512en

this is the level at which management (antimicrobial treatment) and transmission (mixing of fish) patterns can provide a relatively homogenous AMR profile. For the national AMR surveillance, stakeholders (**Annexure I**) identified 24 districts in Pakistan where aquaculture is intensively practised (Table 1). These identified districts exist in every province of Pakistan suggesting the wide spread of aquaculture production in the country. Semi-intensive and intensive farming is commonly practised in the country with Trout fish majorly harvested in the northern areas and Carps in the southern areas of the country. Table 1 gives details for each province of Pakistan. The number of sampling units within these 24 districts will be identified with respect to each production system through snowball sampling (Table 2). Brooder fish farms will not be included in the study.

For the pilot phase, only healthy Trout and Carp fish will be sampled. Trout will be sampled from AJK, GB or KPK whereas Carp will be sampled from Islamabad, Punjab, Sindh or Baluchistan. These fish will only be sampled from intensive farms.

Table 1: AMR Sampling sites, productions systems and common fish species.

Province	Production System	Fish Type	Districts
AJK	Conventional /Capture Inland Semi-Extensive Systems	Trout, Carps	Muzaffarabad, Neelum Area Mirpur Mangla dam
Punjab	Pond Culture (Semi-intensive) Cage Culture (Intensive)	Carps Tilapia	Muzaffargarh Gujranwala Alipur Chatha Sargodha Lilla Faisalabad Mianwali
Sindh	Natural Fish Farming / Intensive	Rohu Thaila Mori Tilapia, Chinese Carp Cat fishes, Indigenous Carps	Sukkur Hyderabad Karachi Badin
Baluchistan	Captured Fisheries Semi-Intensive	Carps Trout	Dera Murad Jamali
GB	Raceway (Intensive)	Trout	Skardu Gilgit Ghizer Jaglot
KPK	Raceway (Intensive) Bio-Flock	Carp Trout	Peshawar Kohat Swat Mardan

Table 2: Production systems and target bacteria for each system.

Production System	Target Bacteria
Inland capture fish (wild-caught fish from rivers)	*Escherichia coli* *Aeromonas* spp.

	Salmonella spp.
Pond culture	*E. coli* *Aeromonas* spp.

3.6 Type of Samples and Collection

3.6.1 Biological Samples

Whole fish samples will be collected directly from the farm level as well as from the fish market, preferable when fresh supplies arrive. The weight of fish should be at least 350 grams and no more than 1 kilogram. Samplers should be familiar with the local operational market details and plan their trips accordingly. For the pilot phase only, biological samples will be collected **(Annexure IV)**.

3.6.2 Environmental Samples

As fish environment is also important so 100ml of water sample per farm will be collected in a pre-labelled sterile container and tested for the same bacterial organisms. The pond water samples will be collected each time along with the collection of regular fish samples. For the pilot phase, no water samples will be collected.

3.7 Logistics of Sampling

Ideally, the first two working days of the week are the most suitable time for the collection and transportation of samples to the laboratory. The number of samples collected and transported to the laboratory will be in accordance with laboratory capacity. A regular sample collection protocol may be helpful in the efficient utilization of laboratory capacity.

3.8 Review of Sampling Methodology and Troubleshooting

The sample collection protocol and AST results will be reviewed annually to identify changes needed to improve the sampling plan for better data quality. For example, less than 70% recovery of *E. coli* isolates of collected samples could be considered as the threshold to make necessary modifications[20] as *E. coli* is a ubiquitous organism and present in diverse food/animal matrices.

3.9 Biosecurity Practices while Collecting Samples

Application of good biosecurity practices while collecting samples is important to avoid transmission of diseases between fish farms and to ensure occupational health and safety. Before visiting a farm/sampling unit make sure that there is no evidence of any infectious disease on that farm/sampling unit. Do not collect samples from a farm/household where there are signs of highly contagious disease in the fish.

Some basic practices may help in improving biosecurity.

1. Minimum number of persons should enter the farm. Ideally, two persons are sufficient-one to collect samples and the second to collect data.
2. The vehicle of the sampler should be parked outside the farm.
3. Proper personal protective equipment (PPEs) such as overall, clean gumboots and gloves must be worn by the sampler and data recorder while entering the farm. It is better to use disposable PPEs which can be disposed of after use on one farm.
4. Follow other biosecurity measures imposed by the farmer/producer.
5. Disinfectant such as potassium peroxymonosulfate may be considered as a disinfectant of choice before collecting the sample in field conditions since it is not inactivated by heat

(normal environmental temperature in tropical locations) or contact with organic matter. Therefore, it remains effective against a range of microorganisms.

6. Once sample is collected and placed in the transport box, the external surface of the container must be disinfected.

3.10 Sample Labelling

Each sample should be disposable placed in a plastic bag pre-labelled appropriately with a permanent marker for proper identification. Relevant epidemiologic information must be obtained on prescribed proforma that should then be placed in a plastic bag and transported to the laboratory along with the biological samples.[19,7] The use of unique identification numbers for ease of labelling (for each farm location) would be considered, for example:

Country code (Pakistan): PK,
Province code (Gilgit-Baltistan): GB,
District code (Gilgit): 11,
Farm Name (Lucky Farm): LF,
Year of sample collection: 2024,
Sample Number: 01,
Combined Identification number: PK/GB/11/LF/24/001.

The information will be collected and recorded in the 'sample collection form' present in **Annexure V**.

3.11 Sample Packaging and Transport

Proper packaging and timely transportation of samples according to international guidelines are not only important for maintaining sample quality and follows biosafety and biosecurity requirements pertaining to the handling of biological materials. Triple-layer packaging is the recommended method of transportation of samples[21,8]. Briefly, a sample should be placed in a leak-proof primary container (a plastic seal bag) covered with absorbent. The second layer should be leak-proof packaging to protect the inner container. The external layer should be durable and hard covering with proper labelling, signs, details of the contents inside and information about the consignee.

The samples should be transported immediately most preferably within 24 hours of collection under cold conditions (at least 4°C) to avoid deterioration of the quality of samples. In the lab, the processing of samples should start immediately and must not exceed 72 hours' post-sample collection. In case of delayed processing, the samples should be stored at 4-8 °C.[9, 21]. The laboratory should keep a record of the time and date of collection of the sample, receipt of the sample in the lab and processing of the sample.

3.12 Laboratory methods

3.12.1 Designated Laboratory for Sample Processing

In Pakistan, the two federal laboratories under the Ministry of National Food Security and Research (MoNFS&R), i.e., the National Reference Laboratory for Poultry Diseases (NRLPD) and the National Veterinary Laboratories (NVL), have been identified as National Reference Laboratories (NRLs) for the animal health sector. Under the Fleming Fund Country Grant, these laboratories have received necessary refurbishments, and capacity strengthening training of staff and have been provided with necessary equipment and consumables for AMR surveillance. Amongst these labs, NVL will be the forefront laboratory that will support Fleming Fund in the initial phases of AMR surveillance in

[21]FAO. 2019. *Monitoring and surveillance of antimicrobial resistance in bacteria from healthy food animals intended for consumption.* Regional Antimicrobial Resistance Monitoring and Surveillance Guidelines – Volume 1. Bangkok.

[22] Smith, P. 2019. The performance of antimicrobial susceptibility testing programmes relevant to aquaculture and aquaculture products. FAO Fisheries and Aquaculture Circular No. 1191. FAO, Rome. http://www.fao.org/documents/card/en/c/ca6028en/

aquaculture along with the Aquaculture and Fisheries Program (AFP) at National Agriculture Research Centre (NARC). AFP will lead the surveillance in aquaculture sector once its fully strengthened to conduct AMR surveillance at national level. There are at least three provincial labs under the Provincial Fisheries departments (two in Punjab and One in Sindh) who will also be on boarded in the implementation of this strategy.

3.12.2 Bacterial Isolation

Samples will be processed following the FAO's Fisheries and Aquaculture Circular (2019) for bacterial isolation[22] and internationally recognized laboratory methodologies, also published by the FAO.

Table 3: List of organs to be targeted for specific bacterial isolation.

Bacteria	Organ	Reference
E. coli	Intestines, gills, mouth	Sichewo, 2013[10]
***Aeromonas* spp.**	Intestines	Daood, 2012[11]; Ashiru et al., 2011[12]
***Salmonella* spp.**	Intestines, gills,	Sichewo, 2013
***Vibrio* spp.**	Intestines, mouth	Sichewo, 2013
***Flavobacterium* spp.**	Spleen, Kidney	Lee, 2023[13]; Saticioglu, 2021[14]
***Yersinia* spp.**	Kidney	Yang, 2021[15]
Listeria monocytogenes	Meat of fish	Quereda, 2021[16]

For the two target bacteria during the pilot phase, in brief, the following routine methodology will be used for the recovery and identification of these target organisms:

- For *E. coli*, swab from intestines will be mixed with buffered peptone water (BPS) (1:10 ratio), incubated for 24 hours at 35◦C ± 1◦C, then a loopful will be streaked onto MacConkey agar followed by incubation at the same temperature above for 19 hours. Lactose fermenting colonies will be transferred into Luria-Bertani (LB) broth again and presumptive *E. coli* will be subjected to routine biochemical tests (Simmon's Citrate, indole tests, etc.) and confirmed using API-20E or (and) MALDI-TOF.

- For *Aeromonas* spp., samples will be cultured using any of the available media (glutamate starch phenol red agar, bile salts-irgnisan brilliant green agar, starch ampicillin agar or non-selective agars such as (tryptic soy agar (TSA) or blood agar). Presumptive isolates will be

[10] Sichewo, P. R., Gono, R. K., Muzvondiwa, J. V., & Sizanobuhle, N. (2013). Isolation and Identification of Pathogenic Bacteria in Edible Fish : A Case Study of Fletcher Dam in. IJSR, 2(9), 269–273.

[11] N. Daood. (2012). Isolation and Antibiotic Susceptibility of Aeromonas spp. From Freshwater Fish Farm and Farmed Carp (Dam of 16 Tishreen, Lattakia). Journal for BASIC SCIENCES Vol. 28, No 1, 27.

[12] A.W. Ashiru, P.O. Uaboi-Egbeni, J.E. Oguntowo and C.N. Idika. Isolation and Antibiotic Profile of Aeromonas Species from Tilapia Fish (Tilapia nilotica) and Catfish (Clarias betrachus). Pakistan Journal of Nutrition 10 (10): 982-986, 2011. ISSN 1680-5194

[13] Lee BH, Nicolas P, Saticioglu IB, Fradet B, Bernardet JF, Rigaudeau D, Rochat T, Duchaud E. Investigation of the Genus Flavobacterium as a Reservoir for Fish-Pathogenic Bacterial Species: the Case of Flavobacterium collinsii. Appl Environ Microbiol. 2023 Apr 26;89(4):e0216222. doi: 10.1128/aem.02162-22. Epub 2023 Mar 28. PMID: 36975784; PMCID: PMC10132118.

[14] Saticioglu IB, Ay H, Altun S, Sahin N, Duman M. 2021. Flavobacterium bernardetii sp. nov., a possible emerging pathogen of farmed rainbow trout (Oncorhynchus mykiss) in cold water. Aquaculture 540:736717. 10.1016/j.aquaculture.2021.736717

[15] Yang Y, Zhu X, Zhang H, Chen Y, Song Y, Ai X. Dual RNA-Seq of Trunk Kidneys Extracted From Channel Catfish Infected With Yersinia ruckeri Reveals Novel Insights Into Host-Pathogen Interactions. Front Immunol. 2021 Dec 15;12:775708. doi: 10.3389/fimmu.2021.775708. Erratum in: Front Immunol. 2022 Jul 27;13:982091. doi: 10.3389/fimmu.2022.982091. PMID: 34975864; PMCID: PMC8715527.

[16] Quereda JJ, Morón-García A, Palacios-Gorba C, Dessaux C, García-Del Portillo F, Pucciarelli MG, Ortega AD. Pathogenicity and virulence of Listeria monocytogenes: A trip from environmental to medical microbiology. Virulence. 2021 Dec;12(1):2509-2545. doi: 10.1080/21505594.2021.1975526. PMID: 34612177; PMCID: PMC8496543.

further characterized using common biochemical tests (oxidase-, catalase+, glucose-fermenting, fermentative on TSA and other characteristics) and identified using API or (and) MALDI-TOF[20].

3.12.3 Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing will be done using disc diffusion method employing international CLSI standards. The list of antimicrobials is given in **Annexure VII**. The antimicrobial panel for Gram-negative organisms would be like those recommended in the literature, following the suggested disc potencies, prescribed QC strains, etc. A customized plate configuration (or similar panel of antimicrobial if using disc diffusion) will be used [17,21]. The panel is typically comprised of antimicrobials critically used in human medicine (public health configuration). The results will be compiled using the form in **Annexure VI**.

4. Coordination Mechanism of Aquaculture AMR Surveillance

The Animal Husbandry Commissioner (AHC) of Pakistan, under the MoNFSR, will be the custodian for all national-level activities related to AMR. MoNFSR will nominate a National Focal Person (NFP) for the surveillance of aquatic animals, who will coordinate the activities with NVL, AFP and Provincial Fisheries Departments, for the implementation of field activities and laboratory testing methods. Provincial departments will nominate a Provincial Focal Person (PFPs) for AMR. These PFPs, in consultation with their department, will identify Field Surveillance Sites (FSSs) and identify Field Focal Points (FFP), who will conduct or facilitate field activities. FFP will coordinate with PFP and NFP for sampling and transport as per the guidelines in this document.

In the pilot phase of surveillance activities, samples will be transported to NVL. However, as resources become available under government or support projects, the capacity of AFP and Provincial Peripheral Laboratories (PPLs) can be enhanced, and samples can be transported to AFP and PPLs for processing and diagnosis. Personnel trained in laboratory procedures will perform primary culture and antimicrobial susceptibility testing following the guidelines in this document within the recommended period. Laboratories should communicate results with the AHC office.

Likewise, data forms accompanying the samples should be entered into an appropriate database like WHONET. Both the laboratory findings and field data should be shared with the Federal Epidemiology Unit (FEU)/AMR-coordination Unit under the AHC office for data management, analysis, and dissemination to relevant stakeholders, including public health counterparts, to foster the One Health

approach for AMR surveillance in Pakistan. This system will be institutionalized for the sustainability point of view.

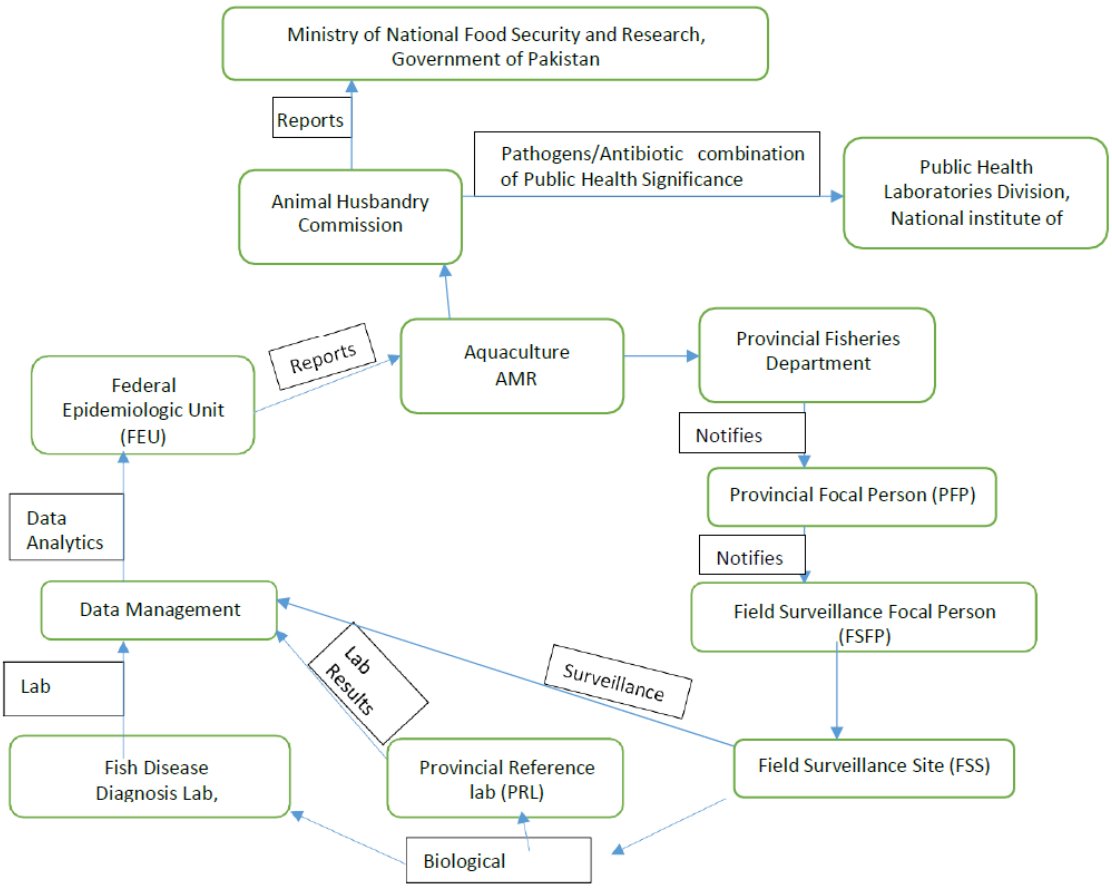


Figure 1: Proposed mechanism of aquaculture surveillance and reporting.

5. Data Management, Analysis and Reporting

5.1 Recording and Storing AMR data

The AMR data should contain essential variables for ease of transition into an AMR analytic software such as WHONET and to be able to contribute to global surveillance initiatives such as the International FAO Antimicrobial Resistance Monitoring (InFARM)[18,23] System. This system is like human health's GLASS, administered by the World Health Organization. The data collected will also be extremely useful for other data utility including detailed epidemiological analysis, for example, risk profiling/risk assessment of aquatic animal products. As described in the WOAH's Terrestrial Animal Health Code, Chapter 6.8, Article 6.8.8 recommends the following for recording and storing AMR data:

- Maintain raw data (primary, non-interpreted), in order to support evaluation of future research questions. This also allows flexibility in conducting retrospective analysis, for example, a change in clinical breakpoints or the utility of both CLSI clinical breakpoints and EUCAST ECOFFs (for the purposes of identifying emerging resistance/non wild-type isolates).
- Consider the interoperability of different computer systems and programs that may be used to exchange surveillance data between platforms.
- Collect quantitative AST results in a national database as the following:
    - distributions of MICs (ug/mL) if using microbroth dilution methodology
    - inhibition zone diameters (mm) if using disk diffusion method

[23] FAO. 2024. The InFARM System. https://www.fao.org/antimicrobial-resistance/resources/infarm-system/en/

- Information that accompanies the AST results, also known as the "metadata" related to the sample or isolate and relevant surveillance demographics:
    - sampling program
    - sampling date
    - production type
    - sample EPID number
    - Type of sample (Fish/water)
    - purpose of sampling
    - type of AST method used
    - geographical origin (geographical information system data, where available) of the farm/ market
    - exposure to antimicrobial agents
    - bacterial isolation rate
- Accompanying laboratory data:
    - laboratory identity
    - isolation date
    - reporting date
    - bacterial species, and, where relevant, other typing characteristics, such as serotype or serovar
    - antimicrobial susceptibility result or resistance phenotype.

5.2 Interpretation of Result

As suggested by the WOAH code and described in FAO guidelines on AMR in aquaculture, the following items should be considered during the interpretation of results [22,23]:

- Report resistant isolates as a proportion of isolates tested, including interpretation criteria when reporting on AMR monitoring and surveillance data in healthy food animals.
- Where possible choose epidemiologic cut-off value (ECOFFS) over clinical susceptibility break-points (CBPs) as the interpretation criteria.
- For ECOFFs, "non-wild type" will only apply to resistant bacterial populations and "wild type" will apply to the normal susceptible population.
- To support tracking of resistance patterns over time, collect data on individual isolates. Include supportive data on the use of antimicrobial and farm management practices.

5.2.1 Analysis

Analysis of various AMR outcomes, in addition to the proportion of resistant isolates should be conducted to gain insight to the data collected. This could be done using commercial analytic softwares (e.g., SAS, Stata, R package), WHONET (preferred) or by participating to the InFARM System (i.e., the private interface enables data analysis and visualizations that could be used in national AMR reporting). The following measurements could be generated:

- AMR outcomes
    - Percentage resistant, intermediate and susceptible (if using CLSI breakpoints) or percentage wild type and non-wild type if using EUCAST Epidemiological Cut-Off values (ECOFF);
    - Percentage of susceptible isolates (exhibited susceptibility to the panel tested) and percentage of multidrug resistant isolates (percentage of isolates resistant to 3 or more classes of antimicrobials in the panel);
    - AMR phenotypic patterns.

- Analytic methods
    - Temporal and geographical variations using appropriate models or descriptive assessment of trends (increased, decreased, stable).

5.2.2 Reporting

The surveillance results and their interpretations will be summarized into various formats such as quarterly bulletins and annual reports. Knowledge dissemination and exchange with specific stakeholders such as the concerned ministries, departments, research institutes, aquaculture producer/farmer network and allied industries (e.g., fish hatcheries, feed mills, pharmaceutical industries) will also be conducted through the AMR-coordination unit at the AHC office. This will ensure and enable stakeholders in timely decision making.

5.3 Contributing to National AMR Integrated Surveillance

The current strategy has been designed to facilitate its incorporation into the proposed revision of AMR National Action Plan (2024-2028) of Pakistan. This strategy is the first of its kind to propose AMR surveillance in aquaculture which is a fast-growing sector in Pakistan that facilitates food security and safety concerns. Through an in-depth and timely intervention through this comprehensive AMR surveillance strategy, MoNFS&R has taken a proactive interest in combating the pandemic of AMR in collaboration with Fleming Fund Country Grant Pakistan.

6. Annexure I – List of Stakeholders at Consultative Workshop

The following is the list of stakeholders who took part in the consultative workshop and provided valuable feedback. These inputs were integrated into the strategy by the contributing authors of the document.

NAME	DESIGNATION	ORGANISATION
Public Sector		
Dr Muhammad Arshad Aziz	Director General	Fisheries Khyber Pakhtunkhwa
Mr. Sadam Hussain	Assistant Director Fisheries	KPKP Fisheries Department
Mr. Rehmat Ali	Deputy Director	Fisheries Department, Gilgit Baltistan
Ghulam Rasool Qambrani	Director (Fisheries Inland)	Livestock, Fisheries & Poultry, Karachi, Sind
Ms. Sana Urooj	Deputy Secretary (Technical)	Forestry, Wildlife & Fisheries Department, Govt of Punjab
Dr. Zahid Sharif	Director Research	Manawa Research Station, Punjab Fisheries, Lahore
Dr Muhammad Ayub	President	Pakistan Fisheries Society
Mr. Shafiq ur Rehman	Deputy Director	Fisheries Department, Punjab, Rawal dam, Islamabad
Dr. SMH Andrabi	Member	ASD, PARC, Islamabad
Dr. Zia Ul Hassan	Director	ASI, NARC, Islamabad
Dr. Hamid Irshad	Principle Scientific Officer	Animal Health Program, ASI, NARC
Dr Reehana Kausar	SSO	AFP, ASI, NARC, Islamabad
Dr. Muhammed Ramzan Ali	PSO/Program Leader	AFP, ASI, NARC, Islamabad
Dr. Khurshid Ahmad	Director General	NVL, MoNFS&R
Dr. Muhammed Abubakar	Senior Research Officer	NVL, MoNFS&R
Dr. Shumaila	Scientific Officer	NVL, MoNFS&R
Dr. Khalid Ashfaq	Deputy Animal Husbandry Commissioner	MoNFS&R (Livestock Wing)
Dr. Usman Ayaz	Assistannt Animal Husbandry Commissioner	MoNFS&R (Livestock Wing)
Mr. Junaid Watto	CEO	Fisheries Development Board, Islamabad, MoNFS&R
Mr. Maratab Ali	Project Director	Fisheries Development Board, Islamabad, MoNFS&R
Private Sector		
Mr. Abid Hussain	CEO	Aquaculture Solutions
Mr. Salahuddin	Director	Salahudin Fish Farms
Ms. Hira Javed	CEO	Tawakal Fisheries, Jhang Road Muzafargarh

Dr. Shahzad Naveed Jadoon	CEO	ALLTECH, Islamabad
Academia		
Dr. Muhammad Akmal Rana	Lecturer	Faculty of Fisheries & Wildlife, University of Veterinary and Animal Sciences, Lahore
Dr. Tahir Usman	Chairman	Department of Livestock Production, College of Veterinary Sciences and Animal Husbandry, Abdul Wali Khan University, Mardan
Dr. Muhammad Zubair Anjum	Assistant Professor	Department of Zoology, Wildlife & Fisheries, PMAS Arid Agriculture Unviersity Rawalpindi
Donor agency		
Dr.Ehtisham ul Haq	Project Coordinator (ECTAD)	Food and Agriculture Organisation
Dr Riasat Wasee ullah	National Project Coordinator (AMR and Food safety)	Food and Agriculture Organisation

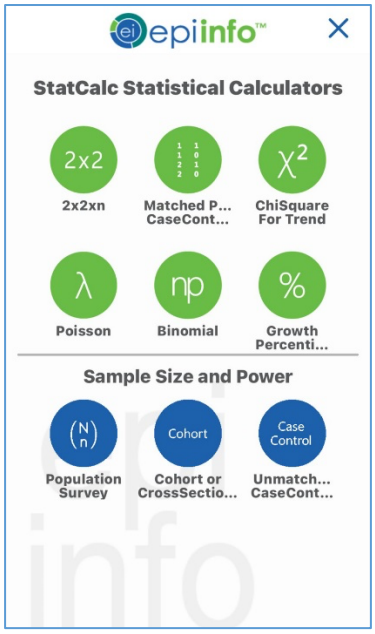
7. Annexure II – Sample size calculation using Epi Info (CDC) for National Surveillance

For sample size calculation using Epi Info (web-tool/mobile app by CDC), the user could either follow the web link (https://github.com/Epi-Info/Epi-Info-iOS/wiki) or download the phone app. The following images represent the interface of the mobile app.

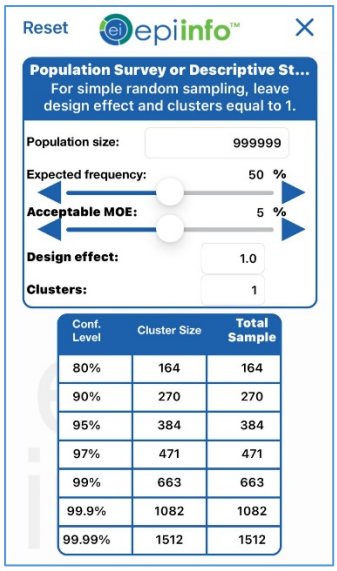
Step 1: Open the app and select the option 'STATCALC'. This will lead the user to sample size calculation options.

epiinfo™
ENTER DATA
Create forms, enter data, browse records, and search the database.
ANALYZE DATA
Visualize analytic results with gadgets, tables, and SQL tools.
STATCALC
Statistical calculators for sample size, power, and more.

Step 2: At this step user must decide the kind of epidemiological study they intend on doing. For the purpose of national surveillance select the option 'Population Survey' (Note that surveillance is not a survey, but this option uses the same methodology for sample size calculation).



Step 3: Now the user must select the population size (number of fish farms in Pakistan), expected frequency of diseases (assumed at 50%), acceptable margin of error (MOE) (5%), design effect (1), and cluster size (1) to determine the sample size. The sample size in the context of the strategy refers to number of isolates required to determine AMR in aquaculture in Pakistan. The selection of appropriate parameters would then lead to the display of sample sizes for different confidence levels. For surveillance it is recommended to use 95% confidence level. The sample size would then be chosen to be 384. Thus the total number of isolates required to assess the AMR levels is 384.



Conf. Level	Cluster Size	Total Sample
80%	164	164
90%	270	270
95%	384	384
97%	471	471
99%	663	663
99.9%	1082	1082
99.99%	1512	1512

Note: the above calculations are subject to change on total number of fish farms in Pakistan, expected frequency of disease based on pilot study, number of clusters that fish farms in Pakistan make.

8. Annexure III - Number of Farms/Point-of-Sale Shops for National AMR Surveillance

The following calculations have been done based on studies done outside of Pakistan. Post pilot phase of surveillance in Pakistan and the data generated therein, the following calculations will be re-adjusted. The tables are only for guiding purpose and do not attempt to finalize sample size required for national surveillance.

Table 4: Number of farms required to be enrolled to estimate the prevalence of AMR in bacterial isolates recovered from fish. Three to five fish per farm will be collected and pooled to form one representative composite sample.

Bacteria	Required number of bacterial isolates to estimate AMR (a)	Expected prevalence (%) of target bacteria in fish samples (b)	Number of samples to be tested to obtain the required number of bacterial isolates c = (a/b)*(100)	Extra samples (d) = [Missingness (5%) + loss of isolates (2%)]*c =	Total number of Fish samples to be tested = c+d
E. coli[24]	384	81.8	469	33	502
Aeromonas spp.[25]	384	19.2	2000	140	2140
Salmonella spp.[26]	384	24	1600	112	1712
Vibrio spp.[25]	384	24.8	1548	108	1657
Flavobacterium spp.	384	50*	768	54	822
Yersinia spp.[27]	384	14	2743	192	2935
Listeria monocytogenes[27]	384	13	2954	207	3161

* Prevalence assumed at 50%.

[24]Sivaraman, G. K., Sudha, S., Muneeb, K. H., Shome, B., Holmes, M., & Cole, J. (2020). Molecular assessment of antimicrobial resistance and virulence in multi drug resistant ESBL-producing Escherichia coli and Klebsiella pneumoniae from food fishes, Assam, India. Microbial pathogenesis, 149, 104581. https://doi.org/10.1016/j.micpath.2020.104581

[25]Zaher, H. A., Nofal, M. I., Hendam, B. M., Elshaer, M. M., Alothaim, A. S., & Eraqi, M. M. (2021). Prevalence and Antibiogram of Vibrio parahaemolyticus and Aeromonas hydrophila in the Flesh of Nile Tilapia, with Special Reference to Their Virulence Genes Detected Using Multiplex PCR Technique. Antibiotics (Basel, Switzerland), 10(6), 654. https://doi.org/10.3390/antibiotics10060654

[26]Traoré, O., Nyholm, O., Siitonen, A., Bonkoungou, I. J., Traoré, A. S., Barro, N., & Haukka, K. (2015). Prevalence and diversity of Salmonella enterica in water, fish and lettuce in Ouagadougou, Burkina Faso. BMC microbiology, 15, 151. https://doi.org/10.1186/s12866-015-0484-7

[27]Terentjeva, M., Eizenberga, I., Valciņa, O., Novoslavskij, A., Strazdiņa, V., & Bērziņš, A. (2015). Prevalence of Foodborne Pathogens in Freshwater Fish in Latvia. Journal of food protection, 78(11), 2093–2098. https://doi.org/10.4315/0362-028X.JFP-15-121

Table 5: Number of farms required to be enrolled to estimate the prevalence of AMR in bacterial isolates recovered in fish environment (water). Water from three-five ponds (from which fish will be collected) will be pooled to form one representative composite sample.

Bacteria	Required number of bacterial isolates to estimate AMR (a)	Expected prevalence (%) of target bacteria in water samples (b)	Number of samples to be tested to obtain the required number of bacterial isolates c = (a/b)*(100)	Extra samples (d) = [Missingness (5%) + loss of isolates (2%)]x c =	Total number of water samples to be tested = c+d
E. coli[28]	384	40	960	67	1027
Aeromonas spp.[29]	384	12.5	3072	215	3287
Salmonella spp.	384	50*	768	54	822
Vibrio spp.	384	50*	768	54	822
Flavobacterium spp.	384	50*	768	54	822
Yersinia spp.	384	50*	768	54	822
Listeria monocytogenes	384	50*	768	54	822

*Prevalence assumed at 50%.

Table 6: Number of point-of-sale shops required to be enrolled to estimate the prevalence of AMR in bacterial isolates recovered from fish.. Three to fish fish of same specie per shop will be collected and pooled to form one representative composite sample.

Bacteria	Required number of bacterial isolates to estimate AMR (a)	Expected prevalence (%) of target bacteria in fish samples (b)	Number of samples to be tested to obtain the required number of bacterial isolates c = (a/b)*(100)	Extra samples (d) = [Missingness (5%) + loss of isolates (2%)]*c =	Total number of Fish samples to be tested = c+d
E. coli[30]	384	92	417	29	447
Aeromonas spp.[31]	384	78.7	488	34	522
Salmonella spp.[30]	384	24	1600	112	1712
Vibrio spp.[30]	384	62	619	43	663
Flavobacterium spp.	384	50*	768	54	822

[28]Chibuike K.U., Iroha I.R., Moses I.B., Chukwunwejim C.R., Peter I.U., Edemekong C.I., Ndugo C.M., Ngene O., Egbuna N.R., Okonkwo-Uzor N.J. Phenotypic Screening of Multidrug-Resistant Escherichia coli from Water and Fish Collected from Different Fish Farms within Abakaliki Metropolis, Nigeria. Sci. Res. Essays. 2021; 16:15–19. doi: 10.5897/SRE2020.6705.

[29]El-Gohary, F. A., Zahran, E., Abd El-Gawad, E. A., El-Gohary, A. H., M Abdelhamid, F., El-Mleeh, A., Elmahallawy, E. K., & Elsayed, M. M. (2020). Investigation of the Prevalence, Virulence Genes, and Antibiogram of Motile Aeromonads Isolated from Nile Tilapia Fish Farms in Egypt and Assessment of their Water Quality. Animals: an open access journal from MDPI, 10(8), 1432. https://doi.org/10.3390/ani10081432

[30]Amin, M. B., Talukdar, P. K., Sraboni, A. S., Islam, M. R., Mahmud, Z. H., Berendes, D., Narrod, C., Parveen, S., & Islam, M. A. (2024). Prevalence and antimicrobial resistance of major foodborne pathogens isolated from pangas and tilapia fish sold in retail markets of Dhaka city, Bangladesh. International journal of food microbiology, 418, 110717. https://doi.org/10.1016/j.ijfoodmicro.2024.110717

[31]Wu, C. J., Ko, W. C., Lee, N. Y., Su, S. L., Li, C. W., Li, M. C., Chen, Y. W., Su, Y. C., Shu, C. Y., Lin, Y. T., & Chen, P. L. (2019). Aeromonas Isolates from Fish and Patients in Tainan City, Taiwan: Genotypic and Phenotypic Characteristics. Applied and environmental microbiology, 85(21), e01360-19. https://doi.org/10.1128/AEM.01360-19

Yersinia spp.[27]	384	28	1371	96	1467
Listeria monocytogenes[27]	384	26	1477	103	1580

*Prevalence assumed at 50%.

9. Annexure IV – Protocols for Pilot AMR Surveillance in healthy fish from Aquaculture farms

For the pilot AMR surveillance study in aquaculture the organisms that are indicator of AMR and of importance as foodborne pathogens, i.e. – *E. coli* and *Aeromonas*, will be the focus. The annexure provides comprehensive guidelines to conduct the Pilot AMR surveillance in aquaculture along with additional information with regards to national surveillance where required. This annexure includes:

- Fish collection techniques
- Fish transportation protocols
- Pre-processing fish prior to dissection
- Dissection of the fish to harvest the organs for bacterial isolation
- Laboratory testing (bacterial culture, isolation, confirmation and antimicrobial resistance testing)
- Data compilation

Collection of Fish from Pond

The initial step in analyzing AMR in fish involves catching the fish from the ponds. For the Pilot AMR surveillance in aquaculture, fish will only be collected from in-land farms that are comprised of multiple ponds. A simple random sampling technique will be utilised to obtain a representative sample of 3-5 fish per farm (epidemiological unit). Farmers will employ one of two randomisation methods to select 3-5 ponds containing the same fish species:

1. Manual Randomisation (Lottery Method): Pond numbers will be written on individual slips, placed in a container, and randomly drawn.
2. Computer-Generated Randomisation: MetaAI on WhatsApp will generate a list of 3-5 random numbers within the range 0-X (X representing the total number of ponds).

One fish will then be collected from each selected pond, ensuring equal probability of selection for all ponds and a representative sample for the study

With respect to fish collection from ponds, it can be done in either active or passive manner. When active gear is moved through the water column to capture fish, the method of fish capture is called ‘active fish collection’, whereas when passive gear is set stationary within the water column to collect fish swimming into it, then it is referred to as ‘passive fish collection’.

For the purpose of the National AMR surveillance strategy as well as the pilot surveillance, active fish collection is suggested for swift and timely collection of fish. Active fish collection is of two types based on pond size. Both methods are explained below.

Active Fish Collection Techniques

Active sampling technique includes seine netting (Figure 2) and cast netting (Figure 3). On fish farms, seine netting and cast netting stand out as two of the quickest and most effective methods for catching fish, offering efficient results with minimal fuss. These are effective on small to medium-sized fish farms with contained or shallow water bodies, where targeting specific species that are concentrated in a particular area is possible. Seine netting can be labor-intensive and may require significant manpower.

It is used in ponds bigger than 0.5-1.0 acres. Cast netting can be skill-dependent and may require experienced operators since it is done in ponds smaller than 0.5-1.0 acres.

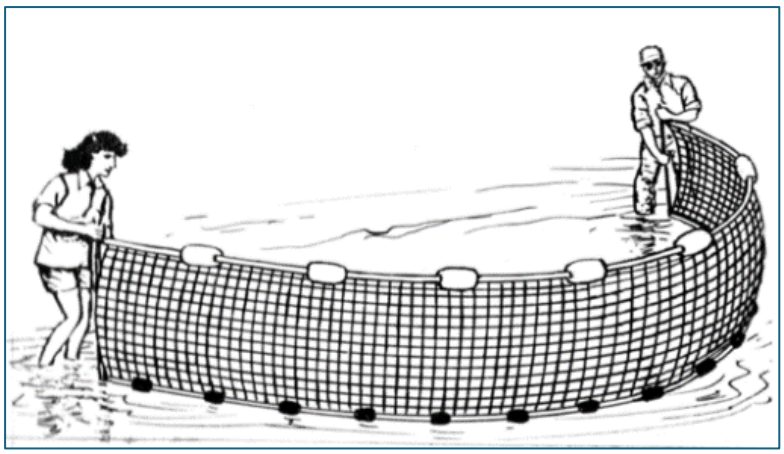


Figure 2: Seine netting for fish capture[22],[23]

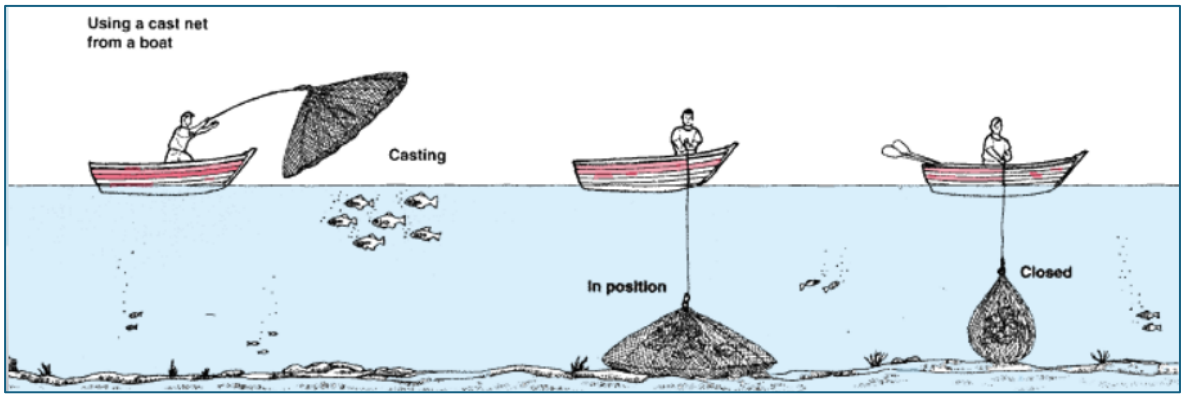


Figure 3: Cast netting for fish capture[24]

Dead Fish Transportation Protocols

The fish once caught must be immediately transported to the laboratory for further processing. For both National surveillance and Pilot surveillance freshly caught, dead fish will be transported whole with guts intact on ice using reverse cold-chain method. The following steps have been primarily adapted from Bonte et al (2008)[25] as well as the FAO's Regional guidelines on Monitoring and Surveillance of AMR, Use and Residues in Food and Agriculture – Volume 3[26]. These steps must be followed to ensure highest possible quality of samples to be received by the laboratory for successful isolation of target bacteria.

- **Step 1: Pre-decide day of sampling and transport:**
  - This should ideally be between Monday to Wednesday, so that the sample could be received from Tuesday till Friday, making sure that sample is never delivered over the weekend when laboratory is not functional.
- **Step 2: Preparation:**
  - Prepare the transport box before capturing the fish. The box with packing material should be laid out at an approachable distance, not so close to get contaminated with water splashes.

[22]https://nebula.wsimg.com/1bcb2f8dab51d3dd7fe4ccfe0f521f56?AccessKeyId=121533AFA5E3032A4431&disposition=0&alloworigin=1
[23] https://www.fao.org/fishery/docs/CDrom/FAO_Training/FAO_Training/General/x6709e/x6709e08.htm
[24] https://www.fao.org/fishery/docs/CDrom/FAO_Training/FAO_Training/General/x6709e/x6709e08.htm
[25] Sample collection, storage and transport from field to reference laboratory. Laurence Bonte, Brigitte Vasset. July 2008. Medecins Sans Frontieres.
[26] FAO 2023, Regional guidelines on Monitoring & Surveillance of AMR, Use and Residues in Food and Agriculture – Volume 3

- The Transport box must be pre-labelled, and vehicle that would transport the sample should be ready-to-go.

- **Step 3: Catch the fish from randomly selected ponds:**
  - Catch the fish as described in the previous section and allow it time to die (30 minutes). The whole fish is required for sampling.
- **Step 4: Packaging**[27]
  - Each individual fish must be placed in a waterproof zip-lock bag. All three-five fish specimens will then be placed in a second bag (composite bag) together. This procedure allows uniformity in the thawing processes since fish aren't iced together in one lump.
  - Place the composite plastic bag of fish in a box container lined with styrofoam with wet ice to maintain the cold chain. This container must be leak- proof, durable and must be able to maintain the cold chain (~4°C).
- **Step 5: Labelling**
  - Seal the box and label with proper signs, and details. The following labelling tag must be used:

Shipper Address	Consignee	Emergency Contact Name & Number	Contents of box
ABC Farms, Union Council, District, Province	National Veterinary Laboratory, Park Road, Chakshehzad, Islamabad	Mr/s. ABC, 0333-0000000	5 fish, Tilapia, 500 g each, 2 kg Ice.

- **Step 6: Farm data information**
  - Each sampler must fill out the sample collection form and place it in a zip lock plastic bag before placing in the sample box. Each pool fish must be labeled on the sample collection form using a unique identification number as described below:
    - Country code (Pakistan): PK,
    - Province code (Gilgit-Baltistan): GB,
    - District code (Gilgit): 11,
    - Farm Name (Lucky Farm): LF,
    - Year of sample collection: 2024,
    - Sample Number: 001,
    - **Combined Identification number: PK/GB/11/LF/24/001**.
  - The sampler with the farmer's assistance must collect the required farm information every month when collecting the sample and record it in the 'sample collection form'.
  - The section 6 of 'sample collection form' will be updated every month when fresh sample is collected.
- **Step 7: Handing over to transport company**
  - The sample must be handed over to the designated transportation company immediately to ensure timely transport to the laboratory.
  - The company must be given details of consignee and emergency contact number. This information should already be printed or written on the label attached to the transportation box.
- **Step 8: Transport**
  - Transport the fish to the laboratory as quickly as possible, ideally within 24 hours.
  - Avoid exposing the fish to extreme temperatures, direct sunlight, or vibration.
  - The person-in-charge of transport at the farm must inform the designated laboratory-in-charge once the sample has been dispatched. The person must also share the tracking

[27] https://www.epa.gov/choose-fish-and-shellfish-wisely/field-sample-handling

number with the laboratory in-charge. This must be done in writing through either a text or WhatsApp text.

Preparation of Fish for Dissection

To accurately collect and analyze fish samples, it is crucial that the person handling them has a thorough understanding of both the external and internal anatomy of fish. For guidance, Figure 4 and Figure 5 illustrate the key anatomical features and structures that the technician needs to be familiar with to ensure effective organ harvesting for successful bacterial isolation.

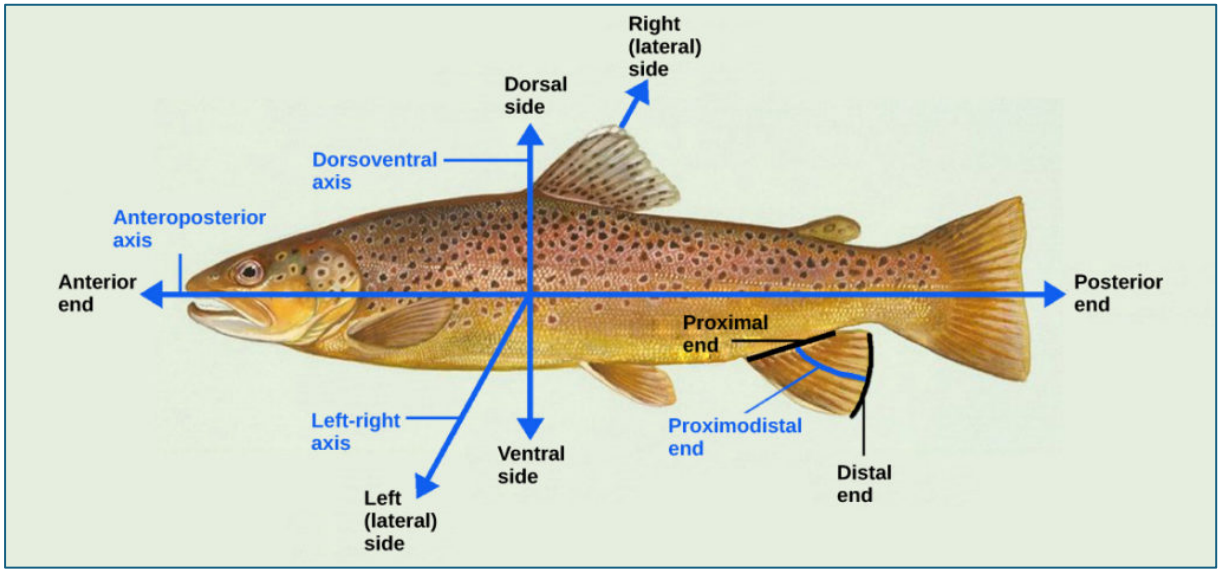


Figure 4: Anatomical planes and directions of a fish

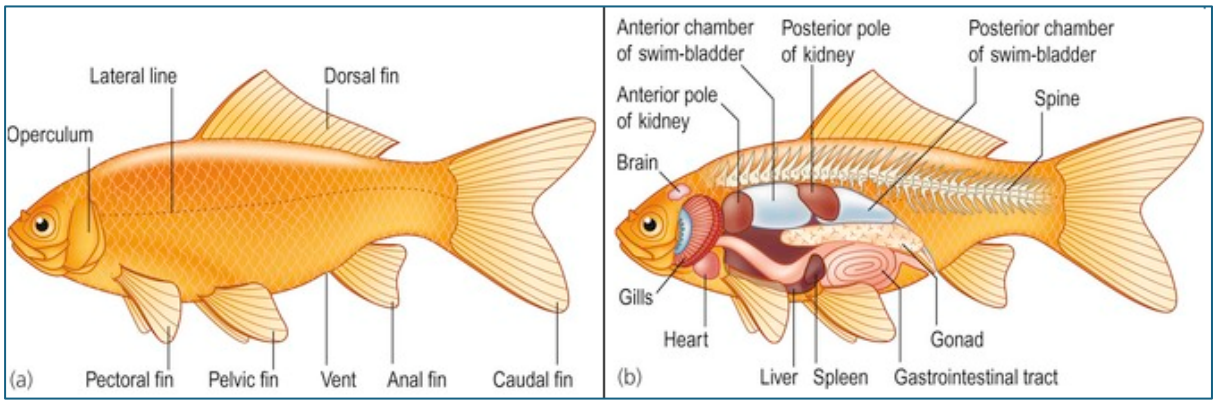


Figure 5: (a,b) External and internal anatomy of a fish, typified by the koi carp (Cyprinus carpio).[28]

Once the anatomy and planes of dissection are clear, the technician can proceed with the disinfection of the fish. Disinfection is a critical step, as it prevents contamination of samples by waterborne bacteria and those present on the skin and internal organs during dissection. To achieve effective disinfection, follow these steps:

1. Ensure not to put pressure on the fish to avoid rupture of the delicate internal organs required for bacterial isolation.
2. Flood the surface with 80% ethanol.
3. Allow the solution to sit until the surface becomes cloudy, ensuring thorough disinfection.

[28] https://veteriankey.com/fish-an-introduction/ accessed on 08/07/2024

4. Wipe clean with gauze.

By following these steps, clean and safe working environment will be ensured before the fish is exposed for organ extraction.

Fish dissection requires technical expertise and a sterile environment to prevent contamination. Once the fish is cleaned with ethanol it is considered ready for dissection. The dissection must be performed in a clean laboratory with pre-sterilised instruments and equipment.

Preparation for Dissection

As soon as the fish is received in the lab it must undergo dissection to harvest the organs for bacterial isolation. This must be done in a laboratory with easy access to sterilised instruments as enlisted in Table 7. The table entails a comprehensive list of prerequisites.

Table 7: List of materials (instruments and accessories) required for dissection of fish.

Sr. No.	Dissection prerequisites	Count	Purpose
1.	Personal protective equipment (PPE) such as gloves, lab coat, and mask	1 per person	Personal protection
2.	Sterile tray	1	To lay the fish
3.	Sterile containers for sample collection	2-3 per farm	To collect organs from fish. Containers will be pre-labelled for each organ. Same organs from 3-5 fish from one farm will be pooled for processing in one single container.
4.	Labeled sampling tubes with appropriate enrichment media	1 per bacterial species	To ensure no cross-contamination
5.	Sterile dissection tools (scissors, scalpels, forceps)	1	To cut and handle the fish
6.	Disinfectant (70% ethanol)	100 ml at least	To disinfect fish surface prior to necropsy.
7.	Access to this document (pdf or hard copy)	1	Guidance if need be
8.	Laboratory data collection forms	1 per farm	Data collection on gross observations

Note that fish from each farm can be handled on the same tray, as long as the tray is disinfected at the end of dissection of each fish.

Dissection

Once the fish is disinfected and laboratory equipment is laid out, then the person in charge must consult Table 3. The table enlists the ideal organs required for isolation of *E. coli* and *Aeromonas*. Only the specified organs need to be harvested for the specific bacteria. For both *E. coli* and *Aeromonas* isolation, intestines must be cut out and its contents be placed in enrichment media for bacterial isolation.

The following is a step-by-step guide adapted[29] for the dissection on Grass Carp (Figure 6) that was caught from the pond at Aquaculture and Fisheries Program at National Agricultural Research Centre at three months of age. The fish weighed 750 grams at the time of dissection.

Figure 6: Grass carp (Ctenopheryngodon idella) caught at Aquaculture Fisheries Program, National Agriculture Research Center, Islamabad to demonstrate dissection and organ removal.

A. **Incision at anus:** To begin, locate the vent (anus) at the ventral side of the fish (**Figure 7**). Note that the vent is situated anterior to the anal fin, on the ventral side of the fish, which is the typical anatomy for most fish species. Then make an incision anterior to the vent of the fish using a fine scalpel blade towards the isthmus.

Figure 7: Locate the vent (red circle) at the ventral side of fish and incising anteriorly towards the isthmus (red arrow).

[29] Web link: https://cpb-us-e1.wpmucdn.com/share.nanjing-school.com/dist/3/28/files/2013/02/fish-dissection-2e5c6ra.pdf accessed on 08/07/2024

Figure 8: Ensure to cut along the mid-ventral line (red and black arrow) without puncturing the organs below.

B. **Extend the cut anteriorly**: Extend the incision along the mid-ventral line, which is the belly of the fish. Take care to make a superficial cut, only penetrating the skin, and avoid damaging the underlying tissue. Extend the cut all the way to the isthmus.

Figure 9: Extend the cut anteriorly going all the way to the isthmus.

C. **Transverse incision perpendicular to isthmus (towards the dorsal surface)**: Proceed by making a transverse incision, situated posterior to the operculum (gill cover), taking care to avoid damaging the underlying organs with excessive depth (Figure 10). This incision should be made across the body, perpendicular to the mid-ventral line. The incision will create a flap, that will allow easy access to all abdominal organs of the fish.

Figure 10: An incision perpendicular to the isthmus and mid-ventral line should be made to create a flap for easy access to organs in the abdomen of the fish.

D. **Reflect the skin flap and scales:** carefully reflect the skin flap and scales laterally using forceps, exposing the underlying tissue (Figure 11). Avoid causing any tears or disruptions to the skin or underlying organs and ensure a gentle and even reflection to both sides.

Figure 11: Expose the organs by reflecting the skin flap.

E. **Open the peritoneal cavity**: Carefully cut through the peritoneum, the thin membrane lining the abdominal cavity, to expose the visceral organs of the fish, such as the liver (1), large intestines (2), swim bladder (3) and the heart (4) that is partially hidden under the operculum in the figure below (Figure 12). The location of the heart close to the gills makes it susceptible to accidental contamination after death, so sampling shortly after death and with aseptic technique are important.

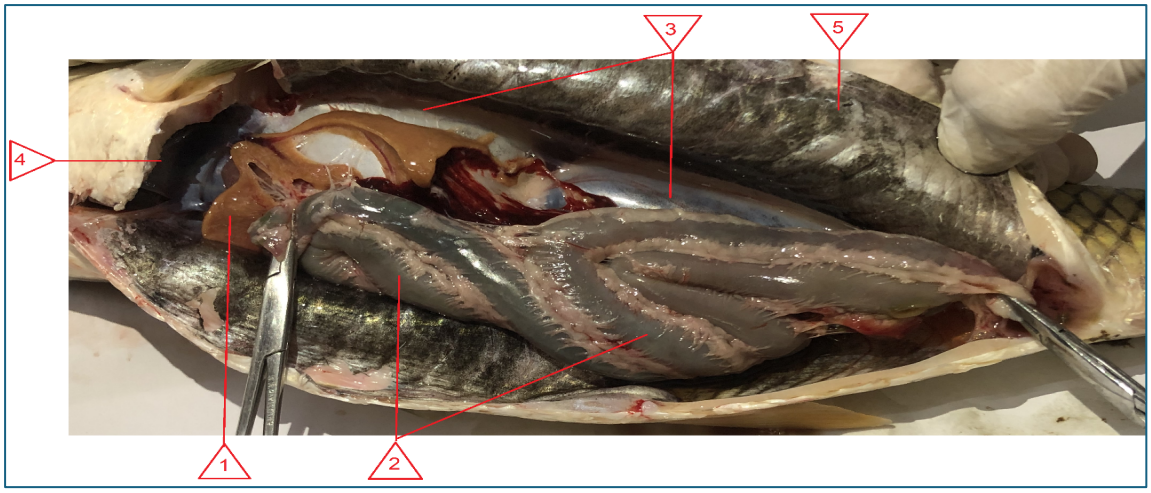


Figure 12: Organs in the abdomen /peritoneal cavity of the fish: liver (1), large intestines (2), swim bladder (3) and the heart (4), Skin flap (5)

F. **Pull aside the gut**: Gently pull aside the gut (2) (esophagus, stomach, intestine and rectum and multiple finger-like pyloric caecae) and spleen (6) to expose the underlying structures (Figure 13). At this stage the intestine must be cut and added to a sterile pre-labelled container and set aside.

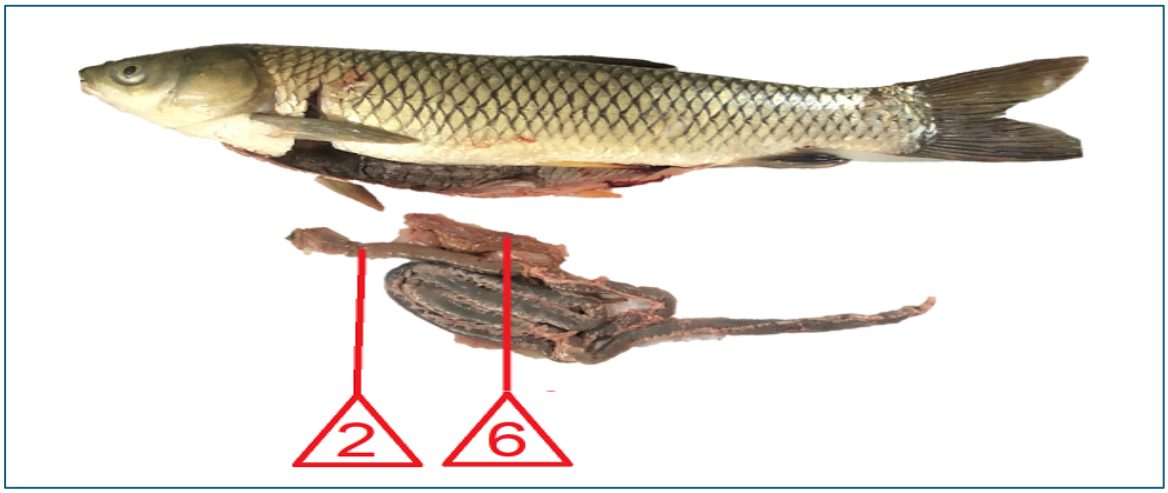


Figure 13: The image shows the intestines and spleen (in the correct orientation) outside the peritoneal cavity of the fish. The intestines are the prime organ of interest for Pilot study, since both E. coli and Aeromonas will be isolated from the intestines.

G. **Accessing spleen**: The spleen (6) is readily accessible as a red ovoid or bilobed organ ventrolateral to the intestinal mass, possibly embedded in abdominal fat (Figure 13). As the spleen is often contaminated during dissection of the fish unless care is taken in exposing the viscera, samples for isolation of bacteria should be collected before completion of the necropsy.

H. **Exposing swim bladder:** The removal of guts will expose the swim bladder (3) (Figure 14). For accessing the kidneys, the swim bladder will have to be removed that will expose the kidney running along the midline of the fish (Figure 15).

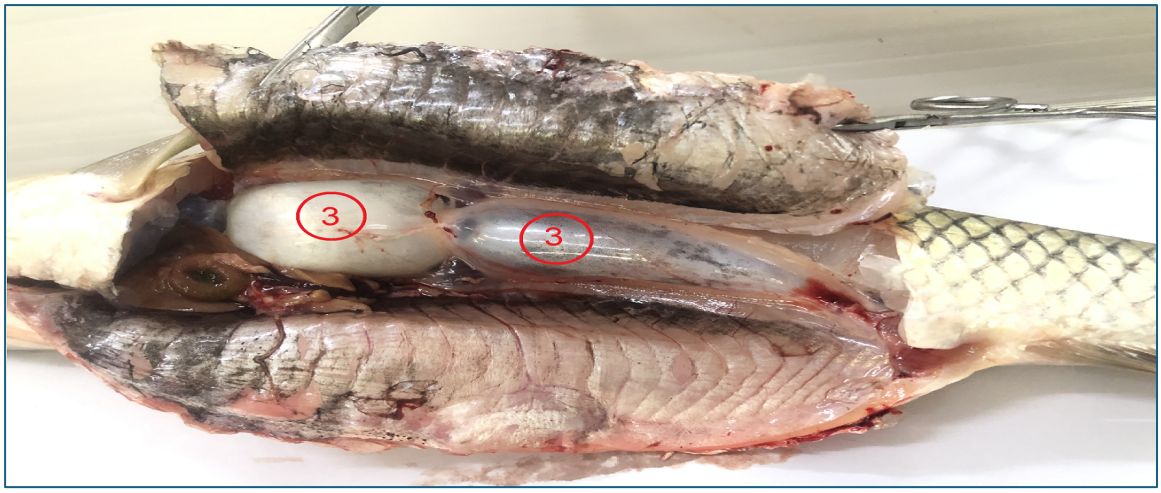


Figure 14: Swim bladder (3) is exposed once the intestines and spleen are removed.

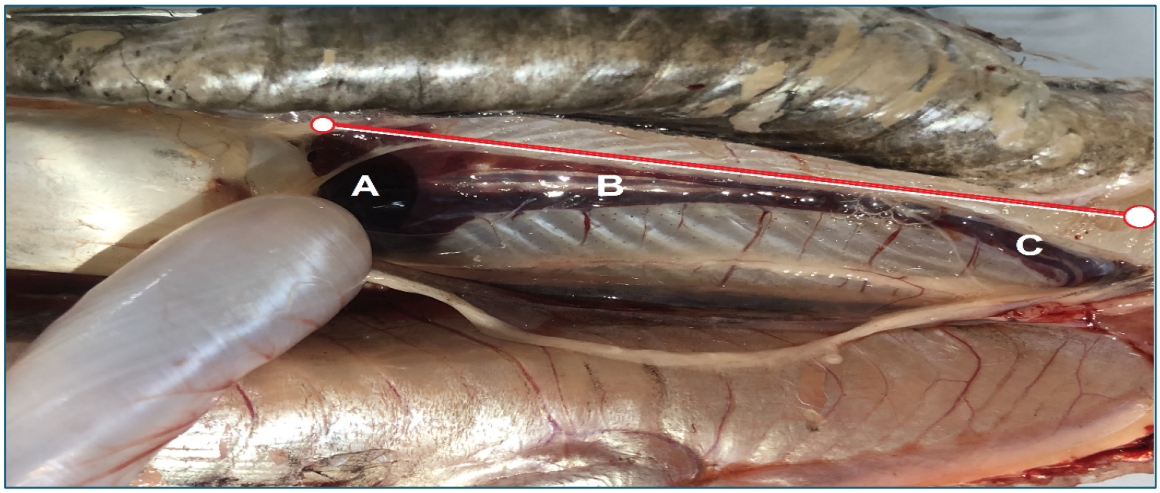


Figure 15: The kidney lies beneath the swim bladder (along the red line). The kidney is divided into A: Head kidney, B: Trunk kidney and C: Posterior kidney.

I. Exposing the heart: Once the organs in the abdominal cavity are removed, the heart (encircled) can be easily accessed and incised out (Figure 16).

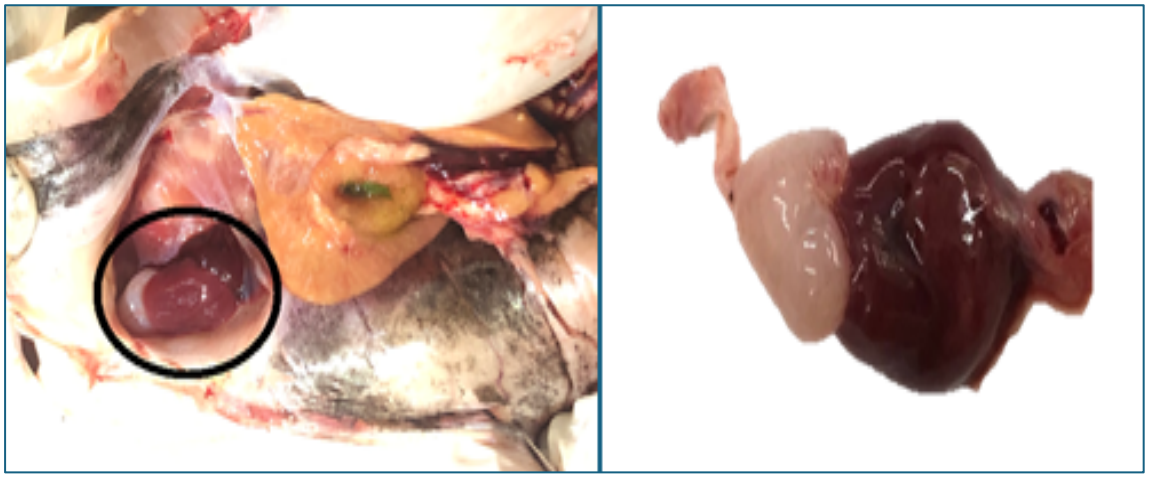


Figure 16: The image on the left shows the heart exposed within the thoracic region. On the right is the image of the heart once it was taken out of the fish.

J. **Exposing gills:** To expose the underlying gills (8) (Figure 17), carefully pull open the operculum (7) and hold with a clamp. Next incise the gills out and place in sample container.

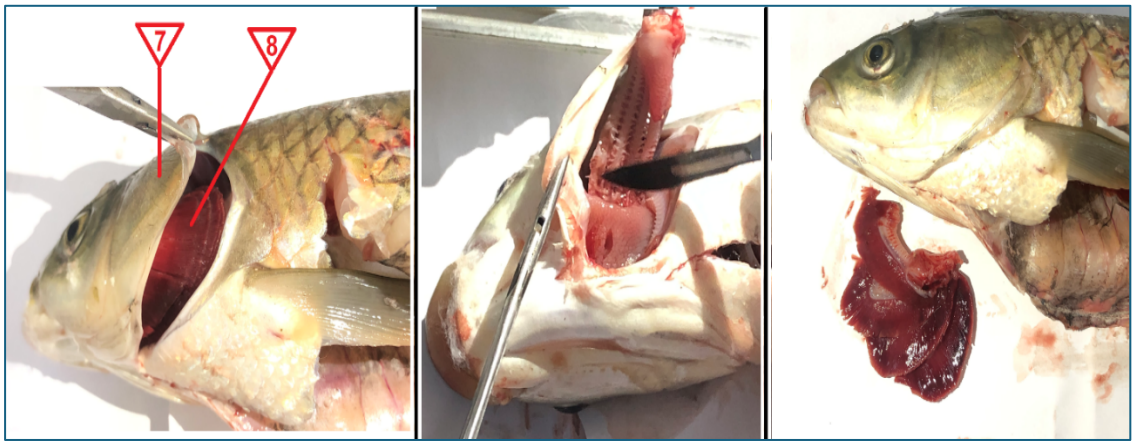


Figure 17: The operculum (7) and gills (8) are shown in the image. The operculum must be retracted to allow exposure of the gills. Once exposed, the gills can be cut out and placed in a sterile container.

The above are guidelines that will facilitate successful harvesting of organs without causing cross-contamination. Once organs are collected, they must be immediately processed for microbiological work within the same day.

▪ Sample Processing

The pooled organ samples once collected will be homogenised in sterile buffered peptone water or another suitable enrichment broth. The following section describes the isolation, confirmation and AMR testing for the priority pathogens- *E. coli* and *Aeromonas* spp.

Bacterial isolation, colony confirmation and antibiotic susceptibility testing for E. coli [30]

In this section details are given for isolation, colony confirmation as well as antibiotic sensitivity testing for *E. coli* from the organs collected in the previous section. In fish, intestines are the preferred organ for *E. coli* isolation.

1. Bacterial isolation of *E. coli*

The isolation of *E. coli* will be conducted using the following protocols.

1.1. Materials Needed for isolation of *E. coli*

1. MacConkey agar
2. Eosin Methylene Blue (EMB) agar
3. Incubator set at 37°C
4. Sterilised plates and loops
5. Weighing balance
6. Autoclave

[30] ISO 16649-2:2001: Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli -- Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide.

1.2. Step-by-step procedure for isolation of *E. coli*

1. Homogenise the fish samples in sterile saline or pre-enrichment Peptone Water Medium.
2. Streak the homogenised and pre-enriched samples onto MacConkey agar and EMB agar plates.
3. Incubate the plates at 37°C for 18-24 hours.
4. Observe for characteristic colonies:
    - On MacConkey agar: Pink to red colonies.
    - On EMB agar: Green metallic sheen colonies.

2. Colony confirmation of E. coli

Once the colonies of *E. coli* are isolated, they must be confirmed using the following biochemical tests.

1. **Indole Test**: Positive (red ring after adding Kovac's reagent).
2. **Methyl Red Test:** Positive (red color after adding methyl red indicator).
3. **Voges-Proskauer Test:** Negative.
4. **Citrate Utilization Test:** Negative.

2.1. Materials Needed for the biochemical tests

- Tryptone broth (for Indole test)
- Methyl Red-Voges Proskauer (MR-VP) broth
- Simmon's citrate agar
- Kovac's reagent
- Methyl red indicator

2.2. Procedure for confirmation of *E. coli*

1. Inoculate respective media with isolated colonies and incubate at 37°C for 24-48 hours.
2. Perform biochemical tests:
    - **Indole Test:** Add Kovac's reagent to the Tryptone broth. A red ring indicates a positive result.
    - **Methyl Red Test:** Add methyl red indicator to MR-VP broth. A red color indicates a positive result.
    - **Voges-Proskauer Test:** Add Barritt's reagents A and B to MR-VP broth. No color change indicates a negative result.
    - **Citrate Utilization Test:** Observe Simmon's citrate agar. No color change (remains green) indicates a negative result.
3. Confirmed colonies must be archived in a biorepository for future use, using the specific SOP designed by the laboratory.

3. Antimicrobial Susceptibility Testing (AST) on *E. coli* isolates

Following successful confirmation of *E. coli*, the bacteria must be tested against the antibiotics (Table 8) using CLSI (CLSI-M100, 33rd Ed) guidelines.

3.1. Materials Needed for AST

- Mueller-Hinton agar plates
- Antibiotic discs (e.g., ampicillin, ciprofloxacin, tetracycline)
- Sterile swabs
- Inoculum standard (0.5 McFarland standard)
- Incubator set at 37°C
- Caliper or ruler for measuring zones of inhibition
- *E. coli* ATCC-25922 (QC Strain)

3.2. Procedure for AST

1. Prepare a bacterial suspension of the confirmed *E. coli* isolate in saline to match the 0.5 McFarland standard.
2. Using a sterile swab, spread the bacterial suspension evenly on Mueller-Hinton agar plates.
3. Place antibiotic discs onto the surface of the inoculated agar using sterile forceps.
4. Incubate the plates at 37°C for 18-24 hours.
5. Measure the diameter of the zones of inhibition around the antibiotic discs using a caliper or ruler.
6. Interpret the results according to the Clinical and Laboratory Standards Institute (CLSI-M100, 33rd Ed) guidelines.

Table 8: List of antibiotics, disc potency and zone diameters for various antibiotics for *E. coli* using CLSI-M100 guidelines

Antibiotics to be tested	Potency (µg)	Susceptible (mm)	Intermediate (mm)	Resistant (mm)
Ampicillin	10	≥17	14-16	≤13
Gentamicin	10	≥18	15-17	≤14
Azithromycin	15	≥13	-	≤12
Cefotaxime	30	≥26	23-25	≤22
Ceftazidime	30	≥21	18-20	≤17
Ciprofloxacin	5	≥26	22-25	≤21
Tetracycline	30	≥15	12-14	≤11
Trimethoprim-sulfamethoxazole	250	≥16	11-15	≤10
Nalidixic Acid	30	≥19	14-18	≤13
Chloramphenicol	30	≥18	13-17	≤12
Imipenem/Meropenem	10	≥23	20-22	≤19
Cefepime	30	≥25	-	≤18

Bacterial isolation, colony confirmation and antibiotic susceptibility testing for Aeromonas spp.[31,32,33,34,35]

In this section details are given for isolation, colony confirmation as well as antibiotic sensitivity testing for *Aeromonas* from the organs collected in the previous section. In fish, intestines are the preferred organ for *Aeromonas* isolation.

1. Bacterial isolation of *Aeromonas* spp.

1.1. Materials needed for isolation of *Aeromonas*

The at materials requires for isolation are enlisted below:

1. Peptone Water Medium Enrichment broth

[31] Janda, J. M., & Abbott, S. L. (2010). The genus Aeromonas: taxonomy, pathogenicity, and infection. Clinical Microbiology Reviews, 23(1), 35-73.
[32] Austin, B., & Austin, D. A. (2016). Bacterial Fish Pathogens: Disease of Farmed and Wild Fish. Springer International Publishing.
[33] CLSI. (2018). Performance Standards for Antimicrobial Susceptibility Testing. 28th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.
[34] Dallaire-Dufresne, S., Tanaka, K. H., Trudel, M. V., Lafaille, A., & Charette, S. J. (2014). Virulence, genomic features, and plasticity of Aeromonas salmonicida subsp. salmonicida. Frontiers in Microbiology, 5, 426.
[35] EUCAST. (2020). The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 10.0.

2. Selective media for *Aeromonas* such as Brilliant Green Agar (BGA), *Aeromonas* Isolation Medium (AIM), or Ryan's medium.
3. Sterilised plates and loops
4. Weighing balance
5. Autoclave

1.2. Step-by-step procedure for isolation of *Aeromonas*

1. Homogenise the fish samples in enrichment broth such as Peptone Water Medium.
2. Streak the enriched samples onto one of the three selective media such as BGA, AIM, or Ryan's medium.
3. Incubate the plates at 28-30°C for 24-48 hours on BGA medium; 28-30°C for 24-48 hours on AIM; and at 28-32°C for 24-72 hours on Ryan's medium.
4. Observe for morphological characteristics of the colonies:
    a. AIM media: Blue colonies
    b. Ryan's medium: Pink Colonies
    c. Brilliant Green Agar: Yellow Colonies

2. Colony confirmation of *Aeromonas*

Once the bacterial colonies are isolated and their morphological confirmation is completed they must be confirmed using the following biochemical tests:

- **Gram Staining:** *Aeromonas* spp. are Gram-negative rods.
- **Oxidase Test:** *Aeromonas* spp. are oxidase positive.
- **Catalase Test:** *Aeromonas* spp. are catalase positive.
- **API 20E System:** Use the API 20E biochemical test strip system for more detailed identification.
- Commercial identification systems.
- MALDI-TOF (NOTE: MALDI-TOF is useful for identification to the genus level, but not to the species level. The Bruker database currently has a limited number of *Aeromonas* spp.) (Perez-Sancho et al., 2018).

3. Antimicrobial Susceptibility Testing (AST) on *Aeromonas* isolates

Following successful confirmation of *Aeromonas*, the bacteria must be tested against the antibiotics (Table 9) using CLSI (CLSI-M45, 33rd Ed) guidelines and *Aeromonas* ATCC 7966 for quality control.

3.1. Materials and Methods

- **Inoculum Preparation:** Prepare a bacterial suspension from pure cultures of Aeromonas, matching the turbidity to a 0.5 McFarland standard.
- **Media:** Use Mueller-Hinton agar for AST.
- **Antibiotic Discs:** Select antibiotic discs based on clinical relevance and guidelines (e.g., tetracycline, ciprofloxacin, ampicillin, and others).
- **Disc Diffusion Method:** Perform the Kirby-Bauer disc diffusion method:
    - Swab the bacterial suspension onto the surface of Mueller-Hinton agar plates.
    - Place antibiotic discs onto the agar surface.
    - Incubate at 35-37°C for 18-24 hours.
- **Interpretation:** Measure the zone of inhibition around each disc and interpret results using Clinical and Laboratory Standards Institute (CLSI-M45, 3rd Ed.) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline, the former is used in Table 9.

Table 9: The table enlists the antibiotics, antibiotic disc potencies and zone diameters against various antibiotics for Aeromonas spp. using CLSI-M45 (3rd Ed.) guidelines.

Types of Antibiotics	Potency (µg)	Susceptible (mm)	Intermediate (mm)	Resistant (mm)
Gentamicin	10	≥15	13-14	≤12
Cefotaxime	30	≥26	23-25	≤22
Ceftazidime	30	≥21	18-20	≤17
Ciprofloxacin	5	≥21	16-20	≤15
Tetracycline	30	≥15	12-14	≤11
Trimethoprim-sulfamethoxazole	1.25/23.75	≥16	11-15	≤10
Chloramphenicol	30	≥18	13-17	≤12

Data Compilation & management

Once the target bacteria have been isolated, confirmed and tested for susceptibility to all antimicrobials in the panel, the data thus generated will be compiled in an excel sheet with a format that aligns with the portal on which it will be submitted and aligned with internationally recognised principles for managing AMR data (Annexe II). Two types of data files will be generated from the data repository for analysis: 1) Recovery or isolation file containing all samples tested and the negative and positive results, and 2) the main AST file containing both the raw inhibition zones in 'mm' and their interpretation according to the CLSI clinical breakpoints or EUCAST clinical or epidemiological cutoffs. Both the recovery and AST data files will contain the epidemiological and other microbiological testing information. Tables in Annex II are for guideline purpose and are subject to change and would consider the inclusion of variables needed for future detailed epidemiological analysis and ease of transition/use of AMR specific software like WHONET and the International Food and Agriculture Organization Antimicrobial Resistance Monitoring (InFARM) System. A data dictionary will be developed corresponding to the variables included in both the bacterial recovery/isolation file and the main AST file. A code list (e.g., species, sample/specimen, location or origin and their description) will follow internationally recognised nomenclature.

Prior to analysis, the AST data will be checked for any errors and validated (resistant, intermediate and susceptible correctly corresponding to the mm zones) prior to analysis using either Microsoft Excel or a commercial software.

Analysis of data

The percentage of bacterial recovery (% positive *E. coli*, % positive *Aeromonas*) will be obtained using the isolation file. For the AST results, common AMR measurements will be obtained including the percentage of resistance to each antimicrobial in the panel, the proportion of resistant, intermediate and susceptible isolates, the number of antimicrobials/classes in the pattern and AMR phenotypic patterns. Results could be stratified by geographical units to assess variations between province, seasonal variations as well as fish species level stratification. The distribution of inhibition zones will also be assessed and used as baseline for detecting shifts in future surveillance years. Analysis will be done using Microsoft Excel, commercial softwares, WHONET or InFARM.

Reporting of data

Results will be visualised in tables and figures and interpreted accordingly, noting key AMR outcomes such as resistance to World Health Organization's Highest Priority Critically Important Antimicrobials (HP-CIA's) and other notable findings (e.g., percentage of multidrug resistant isolates or % susceptible isolates, specific AMR phenotypes) and used in different communication products including those targeting aquaculture producers/stakeholders and for inclusion into the national AMR surveillance

reports or the forthcoming national AMR integrated report for Pakistan and delivered in diverse formats for the broader stakeholder groups.

Conclusion

The establishment of a National AMR Surveillance Strategy for Pakistan's aquaculture sector represents a critical milestone in addressing the escalating threat of AMR. The comprehensive guidelines presented in this document provide a standardised framework for the implementation of the Pilot AMR Surveillance, thereby facilitating the collection of essential baseline data on AMR prevalence in key bacterial diseases affecting the industry. By adopting a coordinated and systematic approach to AMR monitoring, Pakistan's aquaculture sector can effectively mitigate the risks associated with AMR, ensuring the long-term sustainability of the industry while safeguarding public health.

10. Annexure V – Sample Collection Form

SAMPLE COLLECTION FORM

EPID Details

Country:_____

Province:_____

District: _____

Name of Farm (Standard Name): _____

Sample Number: _____

Date of Sample Collection:_____

EPID Number: _____

Name of Laboratory where sample will be processed: _____

Farmer Information

Name:_____

Farm Address:_____

Geo-location (GPS coordinates/IP address):_____

Mobile #:_____

Sampler information:

Name: _____

Mobile #:_____

Designation:_____

Organization:_____

Sample Collection Date:_____

Sample Collection Time:_____

Signature of Collector:_____

Farm/Pond Information

Farm Size (acres): _____

Number of ponds: _____

Fish Type/ Species: _____

Quantity of Fish production at the time of sample collection (Tonnes): _____

Farm History

Date of stocking:_____

Recent Disease Report:_____

Production Type: _ Cage culture/ Conventional/ Capture-inland / Semi-intensive / Extensive/ Pond culture/ Natural Fish-farming / Raceway / BioFlock / others

Sample Details

Sr. No.	Sample ID	Specie/ Common Name	Weight	Fish/ Water Sample	Comments
1.					
2.					
3.					

4.					
5.					

For Laboratory Use Only

Date of sample arrival:_____

Time of sample arrival:_____

Name of person receiving the sample:_____

11. Annexe VI - Microbiological Data Collection Sheet

Table 10: Microbiological data collection forms

A) Whole fish information & demographic data							
Sr.No.	Sample ID	Farm name, address	District	Latitude	Longitude	Fish (scientific name)	Date of sampling from farm
1.							
2.							
Fish & sampling information							
Continued…	Date of organ extraction from fish for bacterial isolation	Sample organ	Pooled count	Size of fish	Weight of fish	Additional notes	
1.							
2.							

B) Isolation of E.coli and confirmation via biochemical tests							
Continued…	Growth on MacConkey (Yes/No) (Pink to red colonies if Yes)	Growth on Eosin Methylene Blue (EMB) agar (Yes/No) (Green metallic sheen colonies if Yes)	Gram staining (+/-)	Indole test (+/-)	Methyl Red Test (+/-)	Voges-Proskauer Test (+/-)	Citrate Utilization Test (+/-)
1.							
2.							

AST of E.coli isolates												
Continued… Only positive isolates will be tested for AST	Ampicillin (mm)	Gentamicin (mm)	Azithromycin (mm)	Cefotaxime (mm)	Ceftazidime (mm)	Ciprofloxacin (mm)	Tetracycline (mm)	Trimethoprim (mm)	Nalidixic Acid (mm)	Chloramphenicol (mm)	Imipenem/Meropenem (mm)	Cefepime (mm)
1.												
2.												

C) Isolation of Aeromonas spp. and confirmation via biochemical tests							
Continued…	Glutamate starch phenol red agar (Yes/No)	Bile salts-irgasan-brilliant green agar (Yes/No)	Selective CromoCen® AGN chromogenic agar medium	Gram staining (+/-)	Oxidase Test (+/-)	Catalase Test (+/-)	API 20E system
1.							
2.							

AST of Aeromonas spp. isolates							
Continued… Only positive isolates will be tested for AST	Gentamicin (mm)	Cefotaxime (mm)	Ceftazidime (mm)	Ciprofloxacin (mm)	Tetracycline (mm)	Trimethoprim-sulphamethoxazole (mm)	Chloramphenicol (mm)
1.							
2.							

12. Annexure VII – List of Antimicrobials

The following are tables derived from the Monitoring and surveillance of antimicrobial resistance in bacterial pathogens from aquaculture Regional Guidelines for the Monitoring and Surveillance of Antimicrobial Resistance, Use and Residues in Food and Agriculture – Volume 3.

Table 11: Adapted from CLSI VET04 (2020b), guidelines. Availability of QC data for suggested panel of antimicrobial agents, disc diffusion (22±2°C, 24–28 h; 22±2°C, 44–48 h; 28±2°C, 24–28 h).

Antimicrobial class	Antimicrobial agent	Disc contents	*Escherichia coli* (ATCC 25922) Disc (MHA)[a]	*Aeromonas salmonicia* subsp. *salmonicida* (ATCC 33658)
Aminoglycosides	Gentamicin	10 µg	✓	✓
Anti-folates	Trimethoprim-sulfamethaxazole	1.25/23.75 µg	✓	✓
β-Lact4am	Ampicillin	10 µg	✓	✓
Macrolides	Erythromycin	15 µg	✓	✓
Phenicols	Florfenicol	30 µg	✓	✓
Quinolones	Enrofloxacin	5 µg	-	✓
	Oxolinic acid	2 µg	✓	✓
Tetracyclines	Oxytetracycline	30 µg	✓	✓

Table 12: Adapted from CLSI VET04 (2020b) guidelines. CLSI Epidemiological cut-off values for aquatic pathogens and agents most frequently used in aquatic animals.

Species	Method	Temperature/ time	Media	Agents
A. salmonicida	Disc diffusion	22°C/44–48 h	MHA	ery, gen, flr, ors, oxo, oxy, sxt
	Broth dilution MIC	22°C/44–48h	CAMHB	flr. oxo, oxy ors
A. hydrophila	Disc diffusion	28°C/24–28 h	MHA	ery, enr, gen, flr, oxo, oxy
	Broth dilution MIC	28°C/24–28 h	CAMHB	ery, enr, gen, flr, oxo, oxy

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